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OBSERVATIONS ON THE USE OF TETRAZOLIUM SALTS IN THE VITAL STAINING OF BACTERIA[1]

L. EIDUS, B. B. DIENA, AND L. GREENBERG

Abstract

Studies on the vital staining of bacteria were carried out to determine the concentrations of tetrazolium salts required for optimal staining, and the amounts which could cause complete inhibition of bacterial growth. Four salts were studied: neotetrazolium chloride (NTC), triphenyltetrazolium chloride (TTC), blue tetrazolium (BT), and nitro-blue tetrazolium (NBT). Evidence has been presented to show that only living, actively metabolizing cells can reduce neotetrazolium to formazan.

Introduction

Tetrazolium salts are being widely used in the biological sciences today. In our laboratory they have been used for determining the count of viable organisms in BCG vaccine (2, 4). In the course of this work, a number of problems were encountered. It was necessary to determine the toxic or inhibitory action of the different salts, the optimum concentration of salts resulting in maximum uptake of dye by the different organisms, the staining pattern and mode of action of the different salts, and, most important of all, whether dead organisms could take up and reduce tetrazolium salts. To investigate these factors more thoroughly the studies were extended to include organisms other than BCG. The results of our observations are reported here.

Procedure

1. Antibiotic and Staining Activity

A modified Sauton (2) and Dubos Tween albumin liquid medium (1) was used for the Mycobacteria and antibiotic assay broth (B.B.L.) for the other organisms. The procedure was to inoculate 0.1 ml of culture into 4.5 ml of medium. For the inoculum, 24-hour cultures of *S. pyogenes*, *E. coli*, *K. pneumoniae*, *M. butyricum*, *M. ranae*, and *M. phlei* and 14-day cultures of *M. avium*, BCG, and the chromogen strains[2] were used. Two hours after the inoculation serial dilutions of tetrazolium salts were added to allow final

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Contribution from the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.
[2]Color-producing atypical Mycobacteria.

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concentrations ranging from 1.25 to 160 μg in the tubes with the Mycobacteria, and from 7.8 to 1000 μg in the tubes with the other organisms. The tubes were incubated at 37° C for periods from 2 to 14 days, depending on the organism studied. They were then observed for visual growth and also for the level at which the maximum uptake of dye occurred.

2. Staining Pattern of the Different Tetrazolium Salts

Two staining procedures were used. The first procedure was that described in earlier reports (2, 4) but using the concentration of dye which, by the technique described above, had been shown to allow for maximum uptake of dye by the organisms concerned. Microscopic examinations were made with and without counterstaining with one part per thousand of acriflavine solution.

The second procedure was as follows: Tubes containing 6.5 ml of antibiotic assay broth were inoculated with 0.1 ml of a 24-hour culture. The tubes were incubated for 36 hours at 37° C and then 0.5 ml of a 0.5% solution of either triphenyltetrazolium chloride or neotetrazolium chloride was added and the tubes were re-incubated for 12 hours at the same temperature. These were then examined microscopically immediately, or after treatment in acetone for periods of from 20 minutes to 12 hours. In both instances, the slides were examined with and without counterstaining with acriflavine. The following organisms were used: *S. pyogenes* (Wood 46, strain 7116 and 1645), *M. flavus*, *S. lutea*, *B. subtilis*, *B. bronchisepticus*, *E. coli* (strain 81, 90), *K. pneumoniae*, *P. vulgaris*, *S. heidelberg*, *S. newport*, *S. enteritidis*, *S. typhi*, *S. montevideo*, *A. aerogenes*, *S. marcescens*, *Sh. flexner*, and *Sh. sonnei*.

3. The Action of Tetrazolium Salts on Dead Organisms

Strains of *M. butyricum* and BCG were heat-killed by autoclaving at 15 lb pressure for 10 minutes, stained by the methods described above using neotetrazolium, and then washed three times in distilled water to remove all traces of free salt. The slides were examined microscopically before and after exposure to ammonium sulphide fumes, which is a strong reducing agent for tetrazolium salts.

Results

1. Antibiotic and Staining Activity

An example of the type of results obtained is shown in Tables I and II. In Table I are shown the minimal concentrations required for three of the salts, neotetrazolium chloride (NTC), triphenyltetrazolium chloride (TTC), and blue tetrazolium (BT), to inhibit completely the growth of the organism. Also shown are the concentrations at which the organisms took up the greatest amount of coloring. In general, it will be noted that TTC is the least toxic of the salts studied, and that the greatest dye uptake by the organisms occurred at a concentration approximately 50% of the toxic level.

Table II shows the results of further toxicity tests on a number of the Mycobacteria with the three salts noted above and a fourth, nitro-blue

tetrazolium (NBT). Here, too, TTC is shown to be the least toxic of the salts and also (not shown in the table) the level for maximum color uptake was found to be approximately 50% of the toxic or inhibitory level. It will be noted that the saprophytic Mycobacteria, *M. butyricum*, *M. phlei*, and *M. ranae*, were sensitive to neotetrazolium, whereas the chromogen strains and *M. avium* were much more resistant.

BCG organisms exhibited a marked increase in sensitivity to all of the tetrazolium salts in Dubos medium, as compared with its sensitivity in Sauton medium. This difference in behavior was not demonstrable with any of the other organisms tested in these two media.

TABLE I

Sensitivity of bacteria to tetrazolium salts

Bacterium	Lowest level required for complete growth inhibition, μg/ml			Level for maximum color uptake, μg/ml		
	TTC*	NTC	BT	TTC	NTC	BT
BCG	125	31.25	31.25	62.5	15.6	15.6
K. pneumoniae	500	62.5	125	250	31.25	62.5
E. coli	>1,000	125	125	1,000	62.5	62.5
S. pyogenes	125	15.6	7.8	62.5	7.8	3.9

*TTC = Triphenyltetrazolium chloride,
NTC = Neotetrazolium chloride,
BT = Blue tetrazolium chloride.

TABLE II

Sensitivity of various Mycobacteria to different tetrazolia

Bacterium	Lowest level showing complete inhibition of growth, μg/ml			
	NTC	TTC	BT	NBT*
Chromogen I				
Sauton	>160	750	80	80
Dubos	>160	1000	80	80
Chromogen III				
Sauton	20	200	20	20
Dubos	20	350	20	160
M. avium				
Sauton	>160	160	>160	>160
Dubos	160	80	>160	160
M. butyricum				
Sauton	10	160	10	10
Dubos	5	80	5	10
M. ranae				
Sauton	2.5	80	2.5	5
Dubos	<1.25	160	10	10
M. phlei				
Sauton	5	80	10	5
Dubos	5	>160	5	10
BCG				
Sauton	40	160	40	40
Dubos	<1.25	40	<1.25	<1.25

*NBT = Nitro-blue tetrazolium chloride.

2. Staining Patterns of the Different Tetrazolium Salts

By microscopic examination, it was found that the different salts have different staining activity. Neotetrazolium penetrates the cell walls, is reduced to formazan, and stains the internal structures. In some organisms, such as *M. butyricum*, the granular bodies are quite evident, whereas in others such as BCG the cells are granular following short periods of exposure to the stain, but are more uniformly stained after 48 hours' exposure and appear larger than they do after acid-fast staining. *M. avium* stained with neotetrazolium displays its usual pleomorphism and the acid-fast chromogen strains show an intense reduction, the individual organisms appear cigar-shaped and are either completely stained or peppered with confluent granules. In cases of both Gram-negative and Gram-positive organisms, the formazan produced by the neotetrazolium remains firmly fixed by the individual cells. Washing in acetone for periods up to 12 hours failed to decolorize them.

Our studies with blue and nitro-blue tetrazolium salts were restricted to the Mycobacteria. The blue tetrazolium did not appear to stain as many of the individual cells as neotetrazolium. The organisms that did take the color, however, were more uniformly stained. The nitro-blue tetrazolium appears very active, causing intense color not only in the organisms but in the surrounding medium as well. Acetone washing was not attempted with these salts.

Triphenyltetrazolium chloride acts somewhat differently than the other salts. The salt apparently is not capable of penetrating the cell walls of the Mycobacteria, and the reduction to formazan occurs only at the surface of the cell. With the chromogen strains the reduction is very intense and large red formazan crystals are formed extracellularly which can be seen macroscopically. Gram-positive (other than the Mycobacteria) and Gram-negative bacteria take up this salt and build up large irregular deposits of formazan. In the case of the Gram-positive organisms, these deposits are formed both inside and outside the cell walls. The Gram-positive bacteria retain their color much more firmly than the Gram-negative organisms. Acetone washing for 12 hours failed to decolorize the Gram-positive, whereas the Gram-negative bacteria were almost completely decolorized in 20 minutes.

Finally, it was noted that with the staining of bacteria with triphenyl-, blue, or nitro-blue tetrazolium salts, formazans of only one color are produced —red with the TTC and blue with BT and NBT—whereas neotetrazolium forms both a reddish and a purple formazan.

3. Action of Tetrazolium Salts on Dead Organisms

Upon microscopic examination, the dead bacteria were found to be unstained. However, after exposure to ammonium sulphide fumes, the organisms appeared light blue in color.

Discussion

A number of problems associated with the vital staining of bacteria have been dealt with. It was found that bacteria do not take up color in the pres-

ence of low concentrations of tetrazolium salts. High concentrations of all of the salts studied were found capable of completely inhibiting bacterial growth. In all cases, optimal staining occurred at concentrations approximately 50% of the inhibitory level. Triphenyltetrazolium chloride was found to be less toxic than either neo-, blue, or nitro-blue tetrazolium salts.

Neotetrazolium was considered to be the most suitable salt for studying enzyme localization. This salt penetrated the bacterial cell walls of all of the species of the bacteria studied more readily than the other salts. Upon counterstaining with acriflavine the cell walls are yellow while the areas of enzyme localization which reduce neotetrazolium appear purple. When reduced, neotetrazolium forms both a red and a purple formazan, while the other salts form only one formazan—either red or blue in color. The ability to form two types of formazan has proved very useful in our work with BCG vaccine. A good metabolically active BCG vaccine stains uniformly and the dominant color is purple, whereas the less active vaccines stain poorly and are more reddish in color.

The tetrazolium compounds do not all exhibit the same staining patterns. Mycobacteria stained with neotetrazolium become granular, stained with blue and nitro-blue tetrazolium they are more uniform, whereas triphenyltetrazolium does not penetrate the cell wall and forms red formazan crystals outside the cell wall. The latter feature has been used by a number of workers (5, 6, 7, 10, 11) for the development of a method for the determination of the viable count of Mycobacteria. The free formazan can be dissolved in acetone and the color concentration used as a rough determination of the bacterial count. Neotetrazolium cannot be used in this manner since the formazan is formed and retained within the cell walls. This latter factor was used in the development of our technique for determining the count of viable organisms in BCG vaccine.

Bacteria, other than Mycobacteria, reduce triphenyltetrazolium salts readily. The Gram-negative organisms appear to take the stain more rapidly than the Gram-positive, but are easily decolorized in acetone. Complete decolorization can occur in 20 minutes. The Gram-positive organisms take the stain more slowly, but the color is retained firmly by the cells. Washing in acetone for periods up to 12 hours will not decolorize them.

The inhibitory action of tetrazolium salts to bacteria has been used as a means for differentiating bacteria. Schönberg (9) and Kraus (8) have developed a differential medium for *E. coli* detection which is based on this organism's high resistance to the salts. Gastambide-Odier and Smith (3) found the chromogens and *M. avium* extremely resistant to neotetrazolium and were able to use this phenomenon in differentiating between these organisms and the other Mycobacteria. In our experience, however, not all chromogenic strains exhibit this high resistance. At least one strain (Table II) has been found to be as sensitive as strains of BCG.

Vitally stained chromogen strains have a number of distinct staining features. When stained with either of neo-, blue, or nitro-blue tetrazolium

salts the formation of formazan is very pronounced and the aggregation within the cell walls causes the organisms to swell to more than twice their normal size. When stained with triphenyltetrazolium, which does not penetrate the cell wall, large deposits of formazan are formed outside the cell and large red shining crystals may be seen suspended in the medium.

To determine whether dead bacteria could take up and reduce tetrazolium salts, strains of *M. butyricum* and BCG were heat-killed, treated with neotetrazolium by our routine methods, and then washed three times in distilled water to ensure that all free traces of salt were removed. Upon microscopic examination the organisms were found to be unstained. After treatment with ammonium sulphide fumes, a strong reducing agent for tetrazolium salts, the organisms became light blue in color. It was evident that the dead cells had adsorbed and fixed the dye, but were not able to reduce it to formazan. Only living, or metabolizing, cells, therefore, can reduce the tetrazolium salts and it is logical to assume that the more active the bacteria the more complete the reduction of the salt.

References

1. DUBOS, R. J. and MIDDLEBROOK, G. Media for tubercle bacilli. Am. Rev. Tuberc. **56**, 334–345 (1947).
2. EIDUS, L., DIENA, B. B., and GREENBERG, L. A rapid method for estimating the bacterial count of BCG vaccine. Can. J. Public Health, **49**, 18–21 (1958).
3. GASTAMBIDE-ODIER, M. and SMITH, D. N. A rapid means of differentiating atypical acid-fast bacilli and avian tubercle bacilli from other Mycobacteria. The neotetrazolium inhibition test. Am. Rev. Tuberc. Pulmonary Diseases, **77**, 662–668 (1958).
4. GREENBERG, L., EIDUS, L., and DIENA, B. B. The vital staining method for the rapid estimation of the bacterial count of BCG vaccine. Am. Rev. Tuberc. Pulmonary Diseases, **78**, 785–787 (1958).
5. KANAI, K. and YANAGISAWA, K. Tetrazolium reduction test as measure to examine total viability of suspensions of tubercle bacilli; studies on techniques of test. Japan. J. Med. Sci. & Biol. **7**, 155–167 (1954).
6. KANAI, K. and YANAGISAWA, K. Tetrazolium reduction test as measure to examine total viability of suspensions of tubercle bacilli; comparison of this biochemical test with culture method. Japan. J. Med. Sci. & Biol. **7**, 249–258 (1954).
7. KOCH-WESER, D. and EBERT, R. H. Tetrazolium reduction by tubercle bacilli. J. Lab. Clin. Med. **46**, 608–614 (1955).
8. KRAUS, H. Zum möglichst sicheren und schnellen Nachweis von coliformen Bakterien und Dyspepsiecoli in Stuhlproben, Milch und Wasser. Zentr. Bakteriol. Parasitenk. **168**, 21–24 (1957).
9. SCHÖNBERG, F. Zum möglichst elektiven Nachweis coliformer Bakterien in Milch, Wasser und Speiseeis auf TTC-Laktose-Agar. Arch. Hyg. u. Bakteriol. **138**, 583–585 (1954).
10. SIMON, C. Beiträge zur Prüfung der BCG–Impfstoffe. Z. Immunitätsforsch. **113**, 45–64 (1956).
11. VANDIVIERE, H. M., GENTRY, W. H., and WILLIS, H. S. A rapid chemical test of total viability for suspension of tubercle bacilli. Am. Rev. Tuberc. **66**, 95–98 (1952).

OXYTOCA GROUP ORGANISMS ISOLATED FROM THE OROPHARYNGEAL REGION[1]

RUDOLPH HUGH

Abstract

An opinion is offered to assign "*Klebsiella*", which are nonmotile, indole positive, and generally gelatinolytic, to the Oxytoca group. The biochemical reactions of 19 strains of the Oxytoca group are described by methods recommended by the Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies for group differentiation within the Enterobacteriaceae. Fifteen of these strains were isolated from the oropharyngeal region of patients with disease.

Introduction

The biochemical behavior pattern of the Klebsiella group has now reached some degree of general widespread agreement (1) and most of the Klebsiella strains isolated fit this pattern. It has become increasingly evident that all coliforms cannot be placed neatly into the groups provided, i.e., Klebsiella, Cloaca, Escherichia, and Citrobacter. To hope that this might be accomplished is probably wishful thinking since the transition from one coliform group to another is gradual. However, there is yet another distinctive group of coliform organisms, the Oxytoca group, which are biochemically homogeneous. They do not fit into any of the above coliform groups for they are nonmotile indole-producing coliforms, which frequently liquefy gelatin and ferment a broader spectrum of sugars than the other coliform organisms. They show some relationship to, but yet are distinct from, typical *Klebsiella*. This group of bacteria is encountered frequently enough to deserve independent taxonomic consideration.

Lautrup (8) has suggested the epithet *Klebsiella oxytoca* for these organisms and traced the history to *Bacillus oxytocus perniosus* (Wyssokowitsch) which was isolated from stale milk in Flugge's laboratory. Malcolm (10) studied 71 strains of *Bacterium oxytocum* isolated from milk and bovine feces with + − + + IMViC reactions. These organisms fermented inositol, adonitol, sucrose, and liquefied gelatin and were recognized to be similar to MacConkey's (9) type 65 isolated from soil, cheese, and human feces. It is apparent that Brooke (2, 3) and Kauffmann (7) considered indole-positive gelatin-liquefying cultures to be "*Klebsiella*". In contrast Edwards and Fife (4) studied a variety of capsulated nonmotile coliforms some of which were both indole and gelatin positive and preferred to retain the genus name *Klebsiella* for nonmotile forms which did not liquefy gelatin. They recognized that this raises a difficult problem since several "*Klebsiella*" capsule types have been and are being established for (*a*) indole and gelatin positive, and (*b*) indole positive and gelatin negative Oxytoca group organisms (2, 12).

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Ørskov (11, 12), Lautrup (8), and Hormaeche and Munilla (6) studied these organisms and isolated strains from feces, urine, sputum, and the respiratory tract. Malcolm (10) found them to occur in relatively low numbers in human and animal feces and considered them to be better adapted to live in soil and water than in the intestine. Ørskov (12) would restrict the application of the epithet *Klebsiella oxytoca*, if indeed it should be used, to strains which form indole, liquefy gelatin, usually dulcitol and *d*-tartrate positive, and methyl red negative. Parr (13) recognized coliforms with + + + + and + − + + IMViC reactions as intermediates, hence separated them from the indole negative *Aerobacter aerogenes*. Henriksen (5) integrated the nonmotile, indole-producing organisms into his concept of "*Klebsiella*" but also observed that such a Klebsiella group lacked homogeneity.

Fifteen Oxytoca group strains were encountered in the oropharyngeal region while investigating the role of infection in the biological behavior of malignant tumors. The biochemical reactions of these organisms are described.

Materials and Methods

The pharyngeal tonsil area was vigorously swabbed with a dry sterile cotton applicator which was then washed in 0.5 ml of sterile infusion broth. A desoxycholate plate was inoculated with a swab wet with this suspension. The plates were incubated at 37° C for 2 days. Red colonies were studied in detail by the described method. The oropharyngeal areas of 820 adults were studied. Two hundred and ninety-seven of these adults were normal individuals obtained from a cancer detection clinic. Each was found free of detectable serious illness as determined by detailed history, complete physical examination, urinalysis, blood count, chest X-ray, serological test for syphilis, proctoscopy, and vaginal cytological studies. The remaining 523 adults were seen in clinics for various diseases. Most of these patients were found to have malignant tumors but they had not recently received antibiotic therapy.

The biochemical methods employed in the identification of these organisms were essentially those recommended by the Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies for group differentiation within the Enterobacteriaceae. The test for urea hydrolysis was performed by inoculating 1 ml of the strongly buffered urea medium of Stuart, van Stratum, and Rustigian (14) with growth scraped from a 20-hour nutrient agar slant culture.

Results

Oxytoca group organisms were not isolated from the 297 normal adults studied. Oxytoca group organisms were isolated from 13* of the 523 patients with disease. It was possible to re-examine 7 of the 13 patients, from whom

*Eight of these 13 patients had histologically verified cancer of the mouth, stomach, or testis, four had stomach pathology (pernicious anemia, achlorhydria, or ulcer), and one had a urinary tract infection.

Oxytoca group organisms were isolated, after intervals varying from 7 days to 15 months (mean 1 month). From 2 of these patients, Oxytoca group organisms were again isolated 7 and 69 days after the first recovery of the organism.

The biochemical properties of these 15 strains and 4 other stock strains of fecal origin* were studied. All 19 strains fermented adonitol, arabinose, dextrose with gas production, inositol, lactose, maltose, mannitol, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Lactose, salicin, and sucrose were uniformly fermented within 24 hours. Positive reactions were obtained with the following tests: indole, Voges-Proskauer, Simmons citrate, KCN, nitrate reduction to nitrites, catalase, and Carlquist lysine decarboxylase. Negative reactions were obtained with the following tests: hydrogen sulphide, motility, nitrate reduction to nitrogen gas, and phenylalanine deaminase. A number of biochemical reactions suggest a diversity of metabolic activities, i.e., methyl red (8 positive, 8 negative, 3 weak orange reactions); dulcitol (9 positive, 10 negative); charcoal gelatin (15 positive, 4 negative); and urea (18 positive, 1 negative).

Eleven strains were urea positive on the first day, 7 positive on the second day, and 1 negative after 2 days' incubation. Charcoal gelatin was liquefied between the 6th and 13th day. Ten strains were positive between the 6th and 8th day of incubation. Four strains failed to liquefy gelatin after 30 days' incubation. Several strains, which were gelatinolytic when isolated, failed to liquefy gelatin after several years. It appears that the urea, gelatin, and methyl red reactions have a tendency to vary from one colony to another within a single strain. The 4 gelatin-negative strains were methyl red and Voges-Proskauer positive and correspond to Ørskov's (12) types 28 and 29. Only 5 strains correspond to her *Klebsiella oxytoca* type 36.

Discussion

The establishment of the Oxytoca group of coliforms does not obscure the integrating nature of the lactose-fermenting organisms. The author would not recommend the perpetuation of the error of past classifications by dignifying each recognizable coliform encountered with a species name. However, by removing the Oxytoca group from the indole-negative Klebsiella group and from the heterogeneous "intermediate" group, attention is focused on the distinctive nature of these organisms. This would contribute toward taxonomic clarification of this frequently encountered group of organisms.

It would appear prudent for the present to place nonmotile indole-producing organisms with variable methyl red and variable gelatin reactions in the Oxytoca group. This would form a group of coliform organisms closely related to Lautrup's and Ørskov's definition of *Klebsiella oxytoca* type 36.

Eight hundred and twenty oropharyngeal areas were studied in an attempt to isolate Oxytoca group organisms. They were not found in the 297 normal individuals studied. It is interesting and surprising to observe that Oxytoca

*These strains were made available to the author by Dr. L. W. Parr of this department.

group organisms were recovered from 13 of the 523 patients with disease. The precise significance of this change in flora has not been elucidated; however one possibility might be considered that it serves as an indicator of a change in the normal physiological state of the patient.

References

1. REPORT of the enterobacteriaceae subcommittee of the nomenclature committee of the international association of microbiological societies. Intern. Bull. Bacteriol. Nomenclature and Taxonomy, **8**, 25–70 (1958).
2. BROOKE, M. The biochemical properties of Klebsiella strains with new capsular antigens. Acta Pathol. Microbiol. Scand. **28**, 328–337 (1951).
3. BROOKE, M. The differentiation of *Aerobacter aerogenes* and *Aerobacter cloacae*. J. Bacteriol. **66**, 721–726 (1953).
4. EDWARDS, P. R. and FIFE, M. Studies on the Klebsiella–Aerobacter group of Bacteria. J. Bacteriol. **70**, 382–390 (1955).
5. HENRIKSEN, S. D. Studies on the Klebsiella group (Kauffmann). III. Biochemical reactions. Acta. Pathol. Microbiol. Scand. **34**, 259–265 (1954).
6. HORMAECHE, E. and MUNILLA, M. Biochemical tests for the differentiation of Klebsiella and Cloaca. Intern. Bull. Bacteriol. Nomenclature and Taxonomy, **7**, 1–20 (1957).
7. KAUFFMANN, F. On biochemical investigations of *Enterobacteriaceae*. Acta Pathol. Microbiol. Scand. **39**, 85–93 (1956).
8. LAUTRUP, H. Gelatin-liquefying Klebsiella strains (*Bacterium oxytocum* (Flugge)). Acta Pathol. Microbiol. Scand. **39**, 375–384 (1956).
9. MACCONKEY, A. Further observations on the differentiation of lactose-fermenting bacilli with special reference to those of intestinal origin. J. Hyg. **9**, 86–103 (1909).
10. MALCOLM, J. F. The classification of coliform bacteria. J. Hyg. **38**, 395–423 (1938).
11. ØRSKOV, I. The biochemical properties of Klebsiella (Klebsiella–Aerogenes) strains. Acta Pathol. Microbiol. Scand. **37**, 353–368 (1955).
12. ØRSKOV, I. Biochemical types in the Klebsiella group. Acta Pathol. Microbiol. Scand. **40**, 155–162 (1957).
13. PARR, L. Coliform bacteria. Bacteriol. Rev. **3**, 1–48 (1939).
14. STUART, C. A., VAN STRATUM, E., and RUSTIGIAN, R. Further studies on urease production by *Proteus* and related organisms. J. Bacteriol. **49**, 437–444 (1945).

DECOMPOSITION OF 2,2-DICHLOROPROPIONIC ACID BY SOIL BACTERIA[1]

LYMAN A. MAGEE AND ARTHUR R. COLMER

Abstract

Eight bacteria capable of decomposing 2,2-dichloropropionate (dalapon) were isolated from soil by means of enrichment techniques and selective media. The decomposition was demonstrated by the clearing of a solid medium containing mineral salts, dalapon, and $CaCO_3$; by a lowering of the pH of a liquid medium containing dalapon as the carbon source; by the increase in chloride ion in the liquid medium; and by the consumption of oxygen by three of the isolates when dalapon was the sole carbon source. Six of these were tentatively classified as *Agrobacterium* and two were tentatively classified as *Pseudomonas*, although there was much overlapping of characteristics. These organisms and many unidentified actinomycetes, molds, and bacteria, including a *Micrococcus* species, overcame the inhibitory effect of dalapon on an agar-decomposing bacterium when grown on the same plate.

Introduction

There is an increasing use of organic chemicals in agriculture. Much work has been done in recent years to determine their fate following introduction into the soil; some investigators have indicated that microbial decomposition is an important factor in their modification. Norman and Newman (8) studied the persistence of some herbicides in soil and found that the addition of organic matter accelerated the disappearance of the herbicides. Audus (1) found evidence that soil bacteria decompose 2,4-dichlorophenoxyacetic acid (2,4-D) and other compounds. Further work on the microbial decomposition of 2,4-D has been reported by Walker and Newman (13) and Rogoff and Reid (9) in 1956 and by Bell (2) in 1957.

Loustalot and Ferrer (6) reported that trichloroacetic acid rapidly disappears from the soil under warm, moist conditions. A kindred chlorinated compound, the sodium salt of 2,2-dichloropropionic acid, dalapon, is commonly used as a herbicide. Many workers have studied the compound and its interrelations with plant life. Southwick (10) found in greenhouse pot trials that 10 lb of dalapon per acre (5 p.p.m.) applied as a sodium salt on a loam soil and watered heavily was not detectable after 4 weeks as determined by successive plantings of susceptible plants. Forty pounds per acre (20 p.p.m.) was still detectable after 8 weeks but not after 12 weeks. He concluded that dalapon is not persistent in most soils under average conditions. Thiegs (12) reported in 1955 that dalapon rapidly disappeared from warm, moist soil, and he attributed this to decomposition by microorganisms. Holstun and Loomis (4) in 1956 described the use of plant bio-assay for determining the loss of dalapon and stated that for most herbicides detoxification by decomposition appears to be primarily a function of microbiological

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Contribution from the Department of Botany, Bacteriology and Plant Pathology, Louisiana State University, and the Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana, U.S.A.

activity. They also stated that the decomposition of dalapon in soils is inhibited by low soil moisture, low pH, large additions of organic matter, and temperatures below 20–25° C, but that these factors were considered to act indirectly by affecting the activity of the microorganism.

In 1957 Jensen (5) described the bacterial degradation of chloro-substituted aliphatic acids. By streaking soil on media containing 0.02 *M* dichloropropionate and excess $CaCO_3$ he was able to isolate five strains of bacteria capable of decomposing the dichloropropionate, as shown by decomposition of the $CaCO_3$ and consequent clearing of the medium around the colonies. These organisms were tentatively assigned to the genus *Agrobacterium*.

This report is a part of a larger study concerned with this microbial decomposition of some herbicides, and deals particularly with the organisms capable of decomposing dalapon.

Materials and Methods

The following mixture (designated as the "basal salts solution") was used in most experiments: K_2HPO_4, 0.08%; KH_2PO_4, 0.02%; $MgSO_4.7H_2O$, 0.02%; $CaSO_4$, 0.01%; Fe (as $FeSO_4.7H_2O$), 0.0001%; Mo (as Na_2MoO_4), 0.00001%; $CaCO_3$, 0.2%; 2,2-dichloropropionate sodium (as dalapon), 0.5% (0.035 *M*); in distilled water. Nitrogen was added either as $(NH_4)_2SO_4$ (0.5%) or $NaNO_3$ (0.5%). In some experiments the medium contained 0.1% yeast extract. For a solid medium, 1.5% agar was added.

The medium for the "associative action" experiment consisted of tryptone, 0.5%; glucose, 0.1%; beef extract, 0.3%; 2,2-dichloropropionate sodium (as dalapon), 0.2% (0.014 *M*); agar, 1.5% in distilled water.

The media were prepared with all ingredients present except the dalapon, bottled in 100-ml amounts, and sterilized by autoclaving. A 10% solution of dalapon was adjusted to pH 7.0 with NaOH and sterilized by filtration through a Millipore filter. The dalapon was added to the medium aseptically just prior to the pouring of the solid medium or just prior to the inoculation of the liquid medium.

Soil which had been treated with dalapon served as the source for most of the dalapon-decomposing organisms. In some instances the Temple (11) perfusion unit was used for enrichment of the cultures. Final isolation of the organism in pure culture made use of the $CaCO_3$–dalapon medium.

Chloride ion in the liquid medium was determined by the Volhard method as described by Willard, Furman, and Bricker (14).

Oxygen uptake by cell suspensions was measured by the conventional Warburg technique. Cells were grown for 48 hours on a reciprocal shaker in the basal salts solution containing 0.5% $(NH_4)_2SO_4$ and 0.1% yeast extract and harvested in a refrigerated centrifuge. The cells were washed twice with the basal salts solution without $CaCO_3$ or dalapon, then suspended in this solution to give an optical density of 1.5 as measured in the Bausch and Lomb Spectronic 20 photometer at 420 mμ.

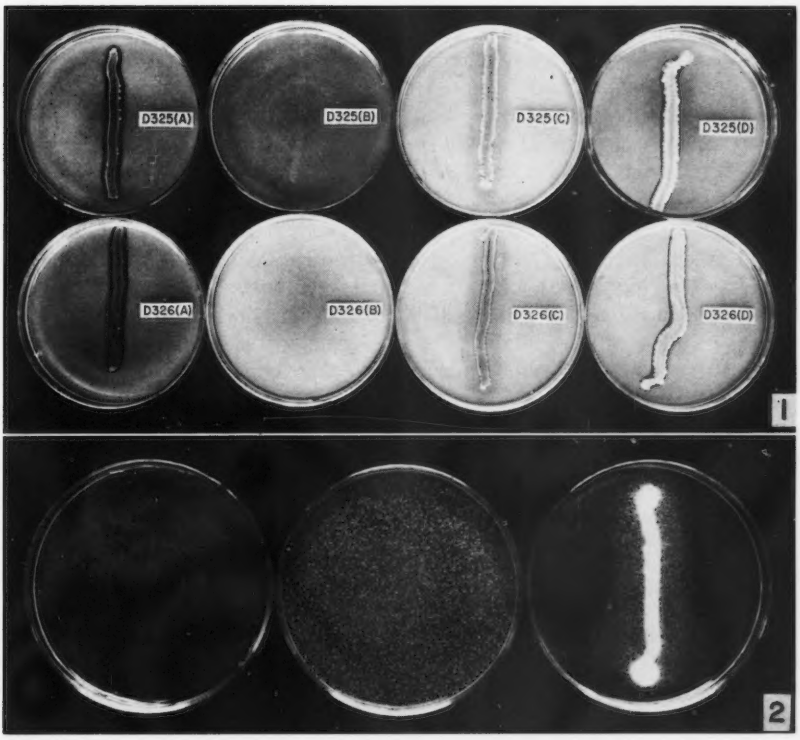

FIG. 1. Growth of two soil bacteria after 7 days on various media containing 5000 p.p.m. (0.035 *M*) dalapon and excess $CaCO_3$. The number on each plate is an arbitrary designation for the isolates used, and the letter in parentheses refers to the medium. Medium A consisted of the basal salts solution with 5000 p.p.m. 2,2-dichloropropionate sodium (as dalapon) as the sole carbon source with 0.5% ammonium sulphate as the nitrogen source. Medium B was the same as medium A except that no dalapon was present. Medium C was the same as medium A except that the nitrogen source was 0.5% sodium nitrate instead of ammonium sulphate. Medium D was the same as medium C except that 0.1% yeast extract was added.

FIG. 2. The associative action of isolate D301 with the agar-decomposing bacterium. Left dish: Dalapon-containing agar seeded with agar-decomposing organism but not streaked with isolate D301. Center dish: Agar-decomposing organism in agar containing no dalapon. Right dish: Dalapon-containing agar seeded with agar-decomposing organism and streaked with isolate D301. The medium was the tryptone, glucose, extract agar with or without 2000 p.p.m. dalapon as indicated. The plates were incubated for 6 days at 30° C.

Results

Isolation of the Organisms

Repeated attempts to isolate dalapon-decomposing organisms from dalapon-treated soil with media containing dalapon and other organic substrates, such as beef extract, peptone, and tryptone, yielded several hundred cultures of bacteria, actinomycetes, and molds that were found to grow readily in the presence of high concentrations of dalapon (up to 10,000 p.p.m.). However, screening procedures showed that most of these failed to grow when dalapon was the sole carbon source in the medium and none of these demonstrated the ability to liberate chloride ion from dalapon.

With the use of the method of Jensen (5) it was found that organisms capable of decomposing dalapon could be readily isolated from the test soil or from the air. Several variations of the basal salts medium were used with either ammonium sulphate or sodium nitrate as the nitrogen source along with dalapon as the carbon source. Some of the isolates produced a clearing of the medium which was evident after 5–6 days and was quite pronounced after about 14 days of incubation. Some of the organisms grew more readily when the medium contained 0.1% yeast extract. The organisms which most readily decomposed dalapon are shown on different media in Fig. 1. The clear zone which formed about the organisms as the $CaCO_3$ was decomposed by the by-products of the dalapon degradation was more pronounced with some isolates than with others.

Decomposition of Dichloropropionate

Flasks containing liquid dalapon medium were inoculated with organisms which were found to clear the solid form of the medium. The cultures were incubated at 30° C for 6 days and then analyzed for chloride ion as a measure of the dalapon decomposed. Since it was found by the Dow Chemical Company (3) that autoclaving dalapon solutions for 50 minutes produces maximum conversion to pyruvic acid and chloride ion, an uninoculated control medium was autoclaved for 1 hour at 121° C and its chloride ion

TABLE I

Decomposition of dalapon by soil microorganisms after 6 days of incubation in liquid medium

Culture	Milli-equivalents Cl^- per ml	% of autoclaved control	Final pH of medium
Distilled H_2O	0.00004	0.06	—
5000 p.p.m. dalapon control, autoclaved 1 hour at 121° C	0.06226	—	1.9
5000 p.p.m. dalapon control, unautoclaved	0.00514	8.25	6.2
D325	0.01840	29.50	5.2
D326	0.01738	27.90	5.4
D327	0.00616	9.88	7.4
D328	0.01636	26.30	5.4
D332	0.01432	23.00	4.9
D334	0.01534	24.60	5.6
D335	0.01738	27.90	5.5
D336	0.01432	23.00	4.9

content was considered to be the total available chloride in the medium. It may be seen from Table I that the unheated, uninoculated control showed a low concentration of chloride ion. All cultures except one showed appreciable liberation of chloride ion. It may also be seen that the final pH of the medium dropped sharply as the dalapon was decomposed.

The oxygen uptake by three isolates with dalapon as the sole carbon source is shown in Fig. 3. Each of the three isolates shown readily utilized glucose as a carbon source and there was a small but significant uptake of oxygen by each isolate when the herbicide was the sole carbon source.

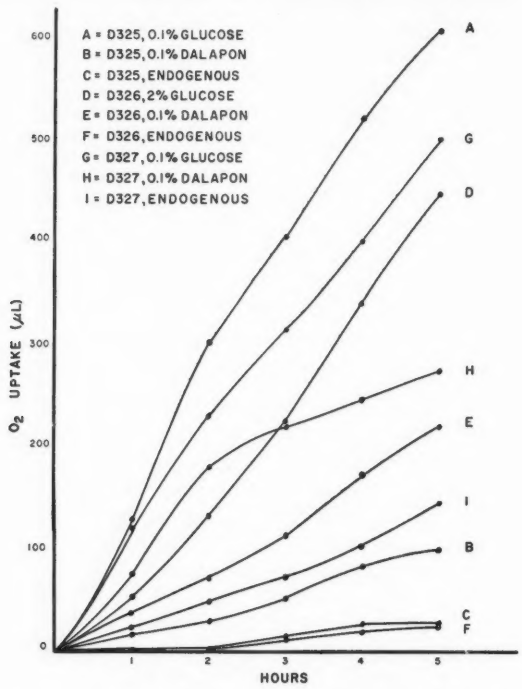

FIG. 3. The oxygen uptake by selected bacteria with dalapon as the sole carbon source.

Associative Action Experiment

During the course of these experiments an agar-decomposing bacterium from the soil was discovered. It was fortuitously found that dalapon in a concentration of 1500–2000 p.p.m. inhibited the growth of this organism, and it was also noted that when an air contaminant (later designated D301) grew on the same plate, a beneficial action occurred for the agar-decomposing bacterium. Figure 2 illustrates this beneficial action. The inhibitory effect of the dalapon for the agar-decomposing bacterium was overcome by the isolate D301 and the originally depressed organism then produced a halo of growth.

Repeated attempts with isolate D301 failed to show that it had the ability to clear $CaCO_3$–dalapon medium and it also failed to grow in media containing dalapon as the sole carbon source.

The associative action procedure was used as a screening test for dalapon decomposition during the early part of this work, but organisms thus isolated failed to show decomposition of the herbicide when tested by other criteria. Numerous bacteria, actinomycetes, and molds demonstrated the ability to overcome the inhibitory effect of dalapon on the agar-decomposing organism (7). Among these were isolates D325, D326, and D328. Isolates D327, D332, D334, and D336 were not so tested.

Identification of the Organisms

The agar-decomposing bacterium isolated from the soil was found to be a highly motile, short Gram-negative rod which was tentatively assigned to the genus *Agarbacterium*. It produced rapid, yellowish-orange, smooth, butyrous growth on nutrient agar and a depression in the agar around each colony was visible after 2–3 days. A medium containing 2% agar inoculated before solidification and incubated with shaking showed rapid growth of the organism and complete decomposition of the agar in less than 7 days (unpublished data). The organism produced no visible growth or change in litmus milk and no hydrogen sulphide from iron citrate agar. Gelatin was not liquefied and starch was not hydrolyzed. No acid or gas was produced from approximately 35 carbohydrates and other carbon compounds tested, including agar.

Isolate D301 was a Gram-positive coccus and was placed in the genus *Micrococcus*.

Isolates D325, D326, D327, D328, D332, and D335 were short Gram-negative rods, motile with peritrichous flagellation. These organisms showed smooth, colorless growth on nutrient agar and on the dalapon medium. The effect on litmus milk was variable, with proteolysis being produced by D325, D326, alkalinity by D327, D332, and D334, and an acid curd by D335. Glucose, sucrose, and lactose were not fermented. Gelatin was liquefied by D325 but not by the others. Starch was not hydrolyzed. There was slight reduction of nitrates by D327 and D328 but not by the other organisms. Indole was not produced. Despite some variations in size and in biochemical reactions, the close similarity of these organisms led to the conclusion that they were probably all different strains of the same organism. Complete classification was not accomplished, but they were tentatively assigned to the genus *Agrobacterium*, with the reservation that some or all of them might be placed in the genus *Alcaligenes*.

Isolates D334 and D336 were short Gram-negative rods, motile with monotrichous flagellation, which showed smooth white growth on nutrient agar and on the dalapon medium. D334 caused alkalinity and D336 caused reduction and slight proteolysis of litmus milk. They did not ferment glucose, sucrose, or lactose, hydrolyze starch, or liquefy gelatin. Nitrates were not reduced and indole was not produced. These isolates were tentatively placed in the genus *Pseudomonas*.

Discussion

The results of these studies confirm the findings of Jensen (5) and lend support to the suggestions of Thiegs (12) and Holstun and Loomis (4) that the rapid loss of 2,2-dichloropropionate (dalapon) from the soil is due to microbial decomposition.

The suggestion of Jensen that the degradation of the chlorinated organic acids occurs by way of a dechlorination process involving a substrate-induced enzyme seems to be a likely one. It may be possible also that some soil microorganisms may hasten the spontaneous hydrolysis of 2,2-dichloropropionate by the utilization and removal of the pyruvate, thus upsetting the equilibrium of the dalapon and water with the products sodium pyruvate and hydrochloric acid. However, since many bacteria are capable of utilizing pyruvate, a relatively large percentage of bacteria should be capable of degrading 2,2-dichloropropionate. Since this circumstance was not found in the study, the substrate-induced enzyme hypothesis of Jensen appears to be the more logical one.

While it is evident from these experiments that some soil bacteria can decompose the herbicide, it also appears that possible inhibitory effects of the herbicide can be circumvented by the growth of tolerant organisms, although these organisms may not attack the foreign chemical itself. The associative action experiment showed that certain organisms, which could not be demonstrated to decompose dalapon, nevertheless overcame the inhibition of the agar-decomposing bacterium by dalapon, presumably by producing some substance(s) that permitted normal growth.

References

1. Audus, L. J. The biological detoxication of hormone herbicides in soil. Plant and Soil, **3**, 170–192 (1951).
2. Bell, G. R. Some morphological and biochemical characteristics of a soil bacterium which decomposes 2,4-dichlorophenoxyacetic acid. Can. J. Microbiol. **3**, 821–840 (1957).
3. Dow Chemical Company. Private communication. 1955.
4. Holstun, J. T., Jr. and Loomis, W. E. Leaching and decomposition of 2,2-dichloropropionic acid in several Iowa soils. Weeds, **4**, 205–217 (1956).
5. Jensen, H. L. Decomposition of chloro-substituted aliphatic acids by soil bacteria. Can. J. Microbiol. **3**, 151–164 (1957).
6. Loustalot, A. J. and Ferrer, R. Studies on the persistence and movement of sodium trichloroacetate in the soil. Agron. J. **42**, 323–327 (1950).
7. Magee, L. A. The responses of some soil microorganisms to sodium 2,2-dichloropropionate and maleic hydrazide diethanolamine. Ph.D. Dissertation, Louisiana State University, Baton Rouge, La. 1958.
8. Norman, A. G. and Newman, A. S. The persistence of herbicides in soils. Proc. Northeastern Weed Control Conf. 7–12 (1950).
9. Rogoff, M. H. and Reid, J. J. Bacterial decomposition of 2,4-dichlorophenoxyacetic acid. J. Bacteriol. **71**, 303–307 (1956).
10. Southwick, L. Progress report on agricultural uses of dalapon. Proc. Northeastern Weed Control Conf. 251–256 (1954).
11. Temple, K. L. A modified design of the Lees soil percolation apparatus. Soil Sci. **71**, 209 (1951).
12. Thiegs, B. J. The stability of dalapon in soils. Down to Earth, **11**, No. 4, 10–12 (1955).
13. Walker, R. L. and Newman, A. S. Microbial decomposition of 2,4-dichlorophenoxyacetic acid. Appl. Microbiol. **4**, 201–206 (1956).
14. Willard, H. H., Furman, H. H., and Bricker, C. E. Elements of Quantitative Analysis. D. Van Nostrand Company, Inc., New York. 1956. p. 128.

A POLYSACCHARIDE ISOLATED FROM CANDIDA ALBICANS AS A GROWTH-PROMOTING FACTOR FOR MYCOBACTERIUM TUBERCULOSIS[1]

E. MANKIEWICZ, E. STACKIEWICZ, AND M. LIIVAK

Abstract

The growth-promoting action of *C. albicans* cells on tubercle bacilli is due, in all probability, to a polysaccharide fraction which has been isolated from yeast cells. A technique for isolating this fraction, some of its chemical characteristics, and its action on tubercle bacilli have been described.

Introduction

The cells, and certain cell-free extracts, of the yeast-like fungus *Candida albicans* have been shown to promote the growth of tubercle bacilli. This fact provided a starting point for the development of a technique for detecting colonies of tubercle bacilli on Loewenstein's medium at an early date. This technique consisted in the superinoculation with a suspension of living *C. albicans* cells of Loewenstein's medium which had been inoculated 3 days previously with the concentrated pathological specimen. *C. albicans* develops its colonies over the sites where tubercle bacilli multiply, thus "tracing" their presence (8, 9). Studies carried out since this method was described have shown that cells, and certain cell-free extracts, of *C. albicans* enhance the rate of multiplication of tubercle bacilli and promote the growth of inocula which contain such small numbers of viable microorganisms that they commonly would fail to yield evidence of *M. tuberculosis* in standard culture media. In addition these studies have disclosed that cells, and certain cell-free extracts, of *C. albicans* promote the growth of streptomycin- and isoniazid-inhibited tubercle bacilli, microorganisms such as are found in specimens obtained from patients undergoing treatment with these agents.

For the following theoretical and practical reasons it seemed desirable to attempt to isolate and to identify the growth-promoting factor in *C. albicans*.

(1) Little is known concerning the normal growth requirements of *M. tuberculosis*. This paucity of knowledge is made evident by the time required for primary isolation of tubercle bacilli on standard culture media, which is still of the order of 4 to 5 weeks. The new method (8, 9) permits detection of tubercle bacilli on Loewenstein's medium in 2 weeks. Incorporation of the *M. tuberculosis* growth-promoting factor in *C. albicans* into standard media appears to offer a means of improving present methods for the isolation of tubercle bacilli.

(2) Very little is known concerning the effects of other microorganisms—bacteria and fungi—on the metabolism of *M. tuberculosis*. Specimens of

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sputum obtained from persons with pulmonary tuberculosis frequently contain fungi (4, 10, 7), especially the *Candida* species, and this suggests that it may be well to explore the possible effects of fungi on tubercle bacilli and vice versa.

(3) Finally, the fact that cells and certain extracts of *C. albicans* possess the ability to promote the growth of streptomycin- and isoniazid-inhibited tubercle bacilli suggests that it may be possible, if only the nature of the growth-promoting factor can be determined, to acquire information concerning the mode of action of these tuberculostatic agents.

Methods Used for the Isolation and Identification of the Growth-Promoting Factor

Preliminary experiments demonstrated that small amounts of *M. tuberculosis* growth-promoting factor are present in media in which *C. albicans* had grown for a period of 2 weeks or more. Microscopic examination of cultures that were 2 weeks old or more disclosed that many of the yeast cells had undergone lysis. It also was found that if washed cells were shaken with glass beads for long periods of time and the cell debris was removed by centrifugation, the supernatant fluid contained growth-promoting factors.

Supernatant fluid, obtained in the manner just described, was deproteinized by several methods, namely, chloroform, ethanol, perchloric acid, ammonium sulphate, and by boiling. Protein-free supernatant still retained growth-promoting factors. This finding clearly indicates that the growth-promoting factor is not likely to be a protein.

The supernatant was extracted with ether, ethyl acetate, acetone, and benzene. These organic solvents failed to remove the growth-promoting factor, which persisted in the inorganic layer. Dialysis of the supernatant against distilled water for 24 hours at 4° C and against running tap water for 24 hours did not remove the growth-promoting factor.

In a buffered medium the growth-promoting factor proved to be heat-stable.

An attempt was then made to determine whether the growth-promoting factor is, or is not, a polysaccharide. The supernatant was deproteinized either by heating to 120° C for 20 minutes, or by ammonium sulphate. The deproteinized supernatant was treated with chloroform. The lipid-free fraction was found to contain the growth-promoting factor. This fraction was dialyzed against running water for 20 hours, and against distilled water for 16 hours. The dialyzate proved to be as active in promoting the growth of small numbers of tubercle bacilli as was the supernatant. Ethanol was added to the dialyzate up to the final concentration of 75%. A cream-colored precipitate formed which was recovered by centrifugation. Water was added and the water-insoluble part removed by centrifugation. The clear solution was again treated with ethanol. The precipitate dissolved readily in water and gave an opalescent, slightly yellow solution. A 10 mg per ml

solution of this precipitate in a solution containing Na_2HPO_4, $MgSO_4$, asparagine, and glycerol allowed the multiplication of very small inocula of tubercle bacilli.

The fraction obtained in the manner just described was found to constitute approximately 10% of the dry weight of cells of *C. albicans*. Analysis showed that it contained less than 2% nitrogen and a watery solution contained only negligible amounts of reducing sugars. When this fraction was hydrolyzed with 0.25 *N* HCl, the amount of reducing sugars appearing during the first 3 hours was small but increased rapidly in the 4th hour. The hydrolysis is not necessarily complete at this point. However, it is apparent that the molecules of reducing sugars appearing in the solution are a result of the hydrolysis of the polysaccharide molecule.

Paper chromatography was used to identify the sugars obtained after hydrolysis. The hydrolyzed solution was passed through Amberlite 1R-4B to remove the HCl, and concentrated to a small volume by evaporation. Aliquots of this were placed on paper strips and descending chromatograms were run in various solvents. Ethyl acetate:acetic acid:water = 3:1:3 v/v, and ethyl acetate:pyridine:water = 2:1:2 v/v were the two solvent systems that gave the best separation and resolution of the spots (3).

Two distinct spots were found and identified as glucose and mannose. The mannose is present in much greater quantity than glucose. The following reagents which give colored reactions with different sugars were used for the identification: aniline phthalate, *p*-anisidine, triphenyltetrazolium, aniline–H_3PO_4, and benzidine. These reagents gave two marked spots. From the results of reactions with molybdate reagents it was concluded that the sugars were not phosphorylated. Ninhydrin did not give any color reaction, thus excluding the presence of amino sugars (2).

The spots were also eluted from the chromatograms and rechromatographed with standard solutions of sugars. It is quite certain that only mannose and glucose were the result of the hydrolysis of the polysaccharide fraction from *C. albicans* cells.

The unhydrolyzed polysaccharide was also chromatographed. It did not give a colored spot with any of the reagents.

As a result of experience gained in the course of the investigation a simple and seemingly effective method was developed for obtaining this compound. The method that presently is being employed is as follows:

C. albicans is allowed to grow on Sabouraud's dextrose agar for 5 days at 37° C. The cells are collected in saline and are washed twice in saline. A heavy suspension in saline is prepared. The dry weight of the cells is determined by measuring the optical density of the saline suspension in a Fisher electrophotometer at 525 mμ and by relating this value to the milligrams dry weight of cells per milliliter as shown on a standard curve drawn from the dry weight of suspensions of known optical density. The suspension in saline then is diluted to a standard density. Following this the cells are shaken in bottles containing glass beads for 4 hours. Cells and cell debris

are removed by centrifugation. The supernatant is heated to a temperature of 120° C for 20 minutes. The precipitate is removed by centrifugation. The supernatant is treated with an equal volume of chloroform. The aqueous portion is dialyzed first against running water and then against distilled water. Following this three volumes of ethanol are added to each volume of dialyzate. A precipitate forms during this process. The precipitate is collected by centrifugation, dissolved in distilled water, and reprecipitated by ethanol. After washing the second precipitate in acetone, it is dried in a desiccator. A yellow, slightly sticky powder is obtained. This powder readily dissolves in saline. Solutions containing 10 to 50 mg of this *M. tuberculosis* growth-promoting compound per ml are used in culture media. These solutions may be exposed to temperatures of 115° C for 15 minutes without significant loss of growth-promoting activity.

Methods Used for Assessing the *M. tuberculosis* Growth-Promoting Effects of Fractions Extracted from Cells of *C. albicans*

Two types of media were used in assessing the growth-promoting effects of extracts of cells of *C. albicans*. One type included "complete" culture media which promote growth at a well-defined and more or less constant rate when inoculated with a certain number of tubercle bacilli. The media of this type which were employed were Dubos-Tween medium, Kirchner's medium with horse-serum, and Loewenstein's medium. The other type included selected ingredients of culture media, substances which provide essential elements for tubercle bacilli if they are to multiply, but which by themselves do not permit growth of these microorganisms. The ingredients used in this type of media were (*a*) saline; (*b*) saline and 2% glycerol; (*c*) saline with 10% horse-serum; (*d*) solutions containing Na_2HPO_4, KH_2PO_4, $MgSO_4$, and sodium citrate; (*e*) solutions as just described with asparagine, or asparagine and glycerol, or horse-serum added.

The fractions extracted from cells of *C. albicans* were added to media of both types in quantities that were equivalent to 10 mg of the dry *C. albicans* cells per ml of medium. All of the tubes of media to which extracts were added were inoculated with approximately the same number of tubercle bacilli. The inoculum consisted of 0.1 ml of a 1 in 10 dilution of a 10-day culture of *M. tuberculosis*—either the human strain $H_{37}Rv$ or the bovine strain BCG—in Dubos-Tween medium.

The *M. tuberculosis* growth-promoting effect of each fraction from *C. albicans* on small inocula of BCG and $H_{37}Rv$ organisms was determined by means of the following:

(1) The rate of growth: Determination of the first macroscopic evidence of growth.

(2) The amount of growth: Colony counts were made when solid media were used. Nephelometric readings were taken when liquid media were employed.

(3) Viability tests: These tests, in which the reduction of neotetrazolium was determined, were carried out at various time intervals, and followed the technique outlined by Vandiviere, Gentry, and St. Willis (12); Winterscheid, Glick, and Mudd (13); Arima (1); with modifications described by Koch-Weser and Ebert (5, 6).

Figure 1 represents the rate of multiplication of a culture of $H_{37}Rv$ grown in the supernatant of crushed *C. albicans* cells. This liquid apparently

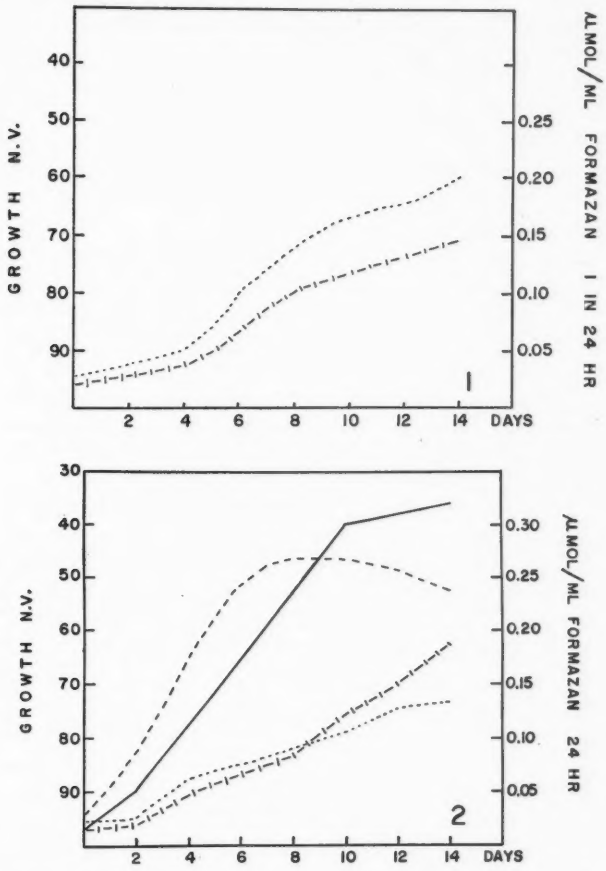

FIG. 1. Growth rate and formazan formation (tetrazolium reduction) of $H_{37}Rv$ in supernatant of crushed *C. albicans* cells.
-|-|-|-|- Growth rate.
– – – – – Formazan formation.
FIG. 2. Growth rate and formazan formation (tetrazolium reduction) of $H_{37}Rv$.
– – – – – Growth rate in medium containing minerals and asparagine.
-|-|-|-|- Formazan formation in this medium.
——— Growth rate after addition of the polysaccharide from *C. albicans*.
— — — Formazan formation in this medium.

contains all the substances necessary for the growth of tubercle bacilli. The rate of growth in this medium is slower than in Dubos-Tween albumin medium, but the reduction of tetrazolium by the microorganisms grown in the supernatant is very active.

Figure 2 represents the rate of multiplication of a small inoculum of $H_{37}Rv$, and the rate of reduction of tetrazolium by these bacilli as they multiply in a medium containing minerals (KH_2PO_4, Na_2HPO_4, $MgSO_4$), glycerol, and asparagine. The addition of the polysaccharides from *C. albicans* to this medium increases the rate of multiplication of the bacteria and the rate at which tetrazolium is reduced.

When added to "complete" culture media (Dubos-Tween albumin medium, Kirchner's medium, or Loewenstein's medium), the polysaccharide fraction of *C. albicans* improved cultural conditions for the laboratory strains $H_{37}Rv$ and BCG in so far as inocula of 10^{-8} mg dry weight of either strain regularly induced growth, whereas 10^{-6} mg were required to initiate growth on the same media without the polysaccharide.

The following observations regarding media containing the polysaccharide fraction from *C. albicans* are of special interest.

(1) One streptomycin-dependent strain requiring a concentration of 100 μg per ml of streptomycin in Loewenstein's medium grew on this medium when 50 mg per ml of the *C. albicans* polysaccharide fraction were incorporated into the medium instead of streptomycin. This observation suggests possible structural similarities between the polysaccharides from *C. albicans* and the streptose in streptomycin which would allow substitution. On the other hand, structural similarities between the streptose from streptomycin and the "lipid-bound" polysaccharide of *M. tuberculosis* have been pointed out by Stacey (11), who suggested "That streptomycin could, therefore, be imagined as a possible blocking group inhibiting the biosynthesis of certain of the tuberculosis polysaccharides". If polysaccharides from *C. albicans* are of a structure similar to the polysaccharides of streptomycin and to those of *M. tuberculosis*, the similarity to the latter might explain the next observation.

(2) Polysaccharides from *C. albicans* stimulate growth of tubercle bacilli contained in specimens from patients undergoing streptomycin treatment. The technique using living *C. albicans* cells to detect microcolonies of *M. tuberculosis* (8, 9) revealed a growth-promoting action of the yeast cells on tubercle bacilli of attenuated viability because of the patients' treatment with chemotherapeutics. The same effect has now been observed when, instead of a suspension of *C. albicans* cells, the polysaccharide fraction from these cells was incorporated into Loewenstein's medium: 20 mg of the polysaccharide dissolved in 0.5 ml saline was added to each Loewenstein's slant and was allowed to dry on the surface of the medium. For the culture of each pathological specimen two such slants were used, together with two control Loewenstein's tubes to which only 0.5 ml of saline were added, to ensure the same degree of humidity. In the 6-month period between January 1st and July 1st, 1958, 322 specimens of sputa or gastric lavages from hospital

patients under treatment with streptomycin and/or isoniazid were cultured in this way. Twenty-one (approximately 6%) of the cultures were positive on both media. In 71 cases, however, only the polysaccharide-containing Loewenstein's media showed macroscopic evidence of *M. tuberculosis* (approximately 22%).

Further studies are under way to relate the metabolic role of the *C. albicans* polysaccharide to deficiencies in tubercle bacilli due to exposure to streptomycin.

References

1. ARIMA, J. Studies on the tetrazolium reducing action of BCG. I. Fundamental experiments on the measurement of the reaction. Japan. J. Tuberc. **2**, 228–237 (1954).
2. BLOCK, R. J., DURRUM, E. L., and ZWEIG, G. A manual of paper-chromatography and paper electrophoresis. Academic Press, Inc., New York. 1955. Chap. VI.
3. JERMYN, M. A. and ISHERWOOD, F. A. Improved separation of sugars on the paper partition chromatogram. Biochem. J. **44**, 402–406 (1949).
4. KAIZAKI, J. Detection of *C. albicans* in patients with pulmonary tuberculosis on culture media. Am. Rev. Tuberc. Pulmonary Diseases, **75**, 836–840 (1957).
5. KOCH-WESER, D. and EBERT, R. H. Tetrazolium reduction by tubercule bacilli. J. Lab. Clin. Med. **46**, 608–614 (1955).
6. KOCH-WESER, D., BARCLAY, W. R., and EBERT, R. H. The influence of isoniazid and streptomycin on acid fastness, tetrazolium reduction, growth and survival of tubercle bacilli. Am. Rev. Tuberc. Pulmonary Diseases, **71**, 556–565 (1955).
7. LAGERCRANTZ, R. Immunological studies of mycobacteria. I. The ability of various bacteria and fungi to induce production of antibodies against erythrocyte-adsorbable antigens from the tubercle bacillus. Acta Pathol. Microbiol. Scand. **38**, 416–422 (1956).
8. MANKIEWICZ, E. *Mycobacterium tuberculosis* and *Candida albicans*: A study of growth-promoting factors. Can. J. Microbiol. **1**, 85–89 (1954).
9. MANKIEWICZ, E. *Candida albicans*: a means for detecting *M. tuberculosis* on culture media. Am. Rev. Tuberc. Pulmonary Diseases, **75**, 836–840 (1957).
10. REIERSOL, S. An investigation of the occurrence of fungi in 250 laryngeal swabs from tuberculosis patients. Acta. Pathol. Microbiol. Scand. **32**, 500–502 (1953).
11. STACEY, M. Polysaccharide components of the tubercle bacillus. Ciba Foundation Symposium. Exptl. Tuberc. Bacillus and Host, **55** (1955).
12. VANDIVIERE, H. M., GENTRY, W. H., and ST. WILLIS, H. A rapid chemical test of total viability for suspensions of tubercle bacilli. Am. Rev. Tuberc. **66**, 95–98 (1952).
13. WINTERSCHEID, L. D., GLICK, M. C., and MUDD, S. The use of neotetrazolium chloride in cultures of tubercle bacilli. Am. Rev. Tuberc. **68**, 625–628 (1953).

IDENTIFICATION OF SERRATIA OCCURRING IN MAN AND ANIMALS[1]

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Abstract

Cultures of *Serratia* even when unpigmented can be identified by their flagella which have an unusual coiled shape. *Serratia* cultures isolated from clinical materials are lactose negative, imvic −−++, sulphide negative, anerogenic, and have a distinctive pattern of fermentative abilities. A different *Serratia*, not so far encountered in clinical materials, is also described. The literature on isolation of *Serratia* from man and animals is summarized.

Isolations of *Serratia* from clinical materials are reported infrequently but continuously. There is no need to trace the early reports except to note that when Bizio first observed these organisms it was in connection with contaminated food (Breed (4)) and that much later Klein (18) also found them in food. Wheat *et al.* (36) reported 11 cases of infections of the urinary tract, 2 of which developed septicemia with 1 ending as a fatal endocarditis. Both the Journal of the American Medical Association (10) and the Lancet (11) have discussed editorially the question of the pathogenicity of *Serratia*. There are 3 reports that associate these organisms with meningitis (1, 20, 26). Hawe and Hughes (15) reported a case of endocarditis apparently due to *Serratia*. Patterson, Banister, and Knight (25) described a case with persistent bacteremia and a fatal termination. Mortelmans (23) stated that Chambon in Viet Nam isolated *Serratia* from the blood of a febrile soldier. Chronic otitis media with *Serratia* infection was reported by Cutin and Sapuppo (7). Mollaret and Chamfeuil (22) described 10 strains from sinusitis pus, 1 from the throat, 1 from urine, and 3 from meat products. Several other reports connect *Serratia* with infection of the respiratory tract. Paine's (24) report of human illness from experimental inhalation of an aerosol of *Serratia* is one. Others are the reports of pseudohemoptysis by Gale and Lord (12) and by Robinson and Woolley (29). To these may be added papers by Böhlck (3) on isolation of *Serratia* during bronchiectasis, and by Vernon and Hepler (32) on the persistent presence of the organism in sputum. Riskó, Nikodemusz, and Tomka (28) found the organism in 3 cases of tuberculosis of knee and hip, apparently as a secondary invader which had however induced increased agglutinins in the patients' blood. Infected urine was reported by Gurevitch and Weber (13), by LoPresti *et al.* (21), and by Khinchuk (17). Waisman and Stone (33) described a case of infant diarrhea with *Serratia* excreted in massive amounts. The ward contamination described by Rabinowitz and Schiffrin (27) suggests that this organism may possess the ability to spread as a hospital infection somewhat as staphylococci do.

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There are some reports of the occurrence of *Serratia* in animals. Bruner (5) identified 1 *Serratia* strain among 676 cultures of enteric bacilli. Deom and Mortelmans (8) isolated the organism at autopsy from blood and organs of a foal dead of sepsis. The species *Serratia anolium* was first isolated from a lizard (9). Capponi *et al.* (6) in a survey of the distribution of enteric bacilli in Viet Nam isolated 6 strains from geckoes and tortoises. Evidently this genus, like other Enterobacteriaceae, may flourish in reptiles ecologically associated with man. Kwapinski (19) observed the organisms as contaminants in a clinical laboratory and attempted to assess the possible danger from their presence.

The present report adds 8 strains collected from clinical materials over a period of several years, namely strains 2690, 2795, 3024, 3224, 3229, 3772, 4060, and 4110 in the list of cultures that follows. These were isolated from urine, pus, feces, and spinal fluid. There is not sufficient clinical and laboratory evidence available to warrant conclusions regarding the etiological significance of these strains. Clinical evaluation will ultimately depend on prompt and accurate recognition of the organism. This is no problem when the cultures present the typical red pigmentation, although the unwary could mistake them for coliform colonies on such media as desoxycholate agar. The real problem is indicated by the fact that unpigmented strains which occurred were all classified under other names until observations to be described were carried out. Chance restoration of pigmentation to one strain which had been listed as a paracolon bacillus led to studies which suggested that the common bacteriological characteristics of *Serratia* would themselves suffice to identify unpigmented strains. Further observations also established that *Serratia* strains of diverse origins and bacteriological characteristics showed a rather unusual type of flagellation.

Cultures

A collection of 21 strains was examined.

2690, abscess, Stinson's gland, North Carolina, 1947
2795, rectal swab, well child, diarrhea ward, Texas, 1948
3024, pus from infected hand, North Carolina, 1948
3224, stock culture labeled paratyphi B, 1951
3229, catheterized urine, Illinois, 1951
3772, feces, adult, Illinois, 1954
3999, variant strain A from No. 4101, 1957
4000, variant strain B from No. 4101, 1957
4001, variant strain C from No. 4101, 1957
4060, child, spina bifida and meningitis, Illinois, 1958
4089, strongly pigmented variant from No. 2690, 1958
4098, stock culture *S. plymuthicum* ATCC 183
4099, stock culture *S. kiliensis* ATCC 992
4100, stock culture *S. marcescens* NCTC 2303

4101, stock culture *S. indica* ATCC 4002
4102, stock culture *S. marcescens* ATCC 8266
4103, stock culture *S. marcescens* ATCC 8285
4104, stock culture *S. urineae* ATCC 11111
4105, stock culture *S. indica* 13341
4106, stock culture *S. anolium* NRRL B-1700
4110, cancer of buccal cavity, D. C., 1958

Morphology

All of the strains studied were Gram-negative bacilli. Cell lengths varied within strains and some, such as *S. plymuthicum* and *S. kiliensis*, formed numerous filaments. Three of the 21 strains were nonflagellated. The other strains were well flagellated, having peritrichous flagella with a characteristic *coiled* shape which is shown in Figs. 1 to 6. This distinctive shape of the flagella was present on strains labeled with 5 species names, namely *S. marcescens* (Figs. 1 and 2), *S. plymuthicum* (Fig. 3), *S. kiliensis* (Fig. 4), *S. anolium* (Fig. 5), and *S. indica* (Fig. 6). The culture received as "*S. indica* 4002" showed two types of individuals. One had typical coiled flagella. The other has flagella of more normal shape, as shown in Fig. 7. The type with normal flagella was isolated in pure culture, had bacteriological characteristics in agreement with the other form, and appeared to be a stable variant. While the coiled shape of flagella has been seen randomly in certain other bacteria, in none of the others has the coiled shape been predominant. In this respect the coiled flagellation of *Serratia* appears to be unique.

Bacteriology

Fermentative and other characteristics were determined by slight modifications of the methods of Sigtenhorst (31). There were two definite groups among the 21 strains, which will be referred to as *Pattern 1* and *Pattern 2*.

Of *Pattern 1*, 17 strains were studied. These formed acid promptly from adonitol, aesculin, cellobiose, galactose, glucose, glycerol, inositol, levulose, maltose, mannitol, mannose, salicin, sorbitol, sucrose, and trehalose. No acid was formed in arabinose, dextrin, dulcitol, erythritol, inulin, lactose, melezitose, melibiose, methylglucoside, raffinose, rhamnose, and xylose. No gas was formed. Acetylmethylcarbinol, citrate, gelatin, motility, and nitrite tests were positive. Urease, indole, methyl red, sulphide, 10% lactose slant, nitrogen gas, and phenylpyruvic acid tests were negative. All strains grew both aerobically and anaerobically with glucose in the Hugh–Leifson oxidation–fermentation medium. Freshly isolated strains often failed to show pigment.

Only four strains fell into *Pattern 2*. Acid was formed in aesculin, arabinose, cellobiose, dextrin, erythritol, galactose, glucose, glycerol, inositol, lactose, levulose, maltose, mannitol, mannose, melezitose, melibiose, methylglucoside, raffinose, salicin, sorbitol, sucrose, trehalose, and xylose. No acid was

formed in adonitol, dulcitol, inulin, or rhamnose. A small amount of gas was formed from glucose. The 10% lactose slant was positive. Also positive were the acetylmethylcarbinol, citrate, motility, and nitrite tests. Urease, indole, methyl red, gelatin, sulphide, nitrogen gas, and phenylpyruvic acid tests were negative. All strains fermented glucose in the Hugh–Leifson oxidation–fermentation medium. Pigment was not generally evident on nutrient agar but apparent after storage in the cold on heart infusion agar.

Antibiotics

Young broth cultures were swabbed on plates of nutrient agar and commercial antibiotic disks added manually, employing strong concentrations only. After overnight incubation the presence or absence of an easily seen zone of inhibition was recorded. There was no discernible difference between strains of the two patterns. None of the 21 strains was inhibited by bacitracin, penicillin, or cathomycin. All were inhibited by neomycin and by chloramphenicol. The numbers of strains inhibited by the other antibiotics were: aureomycin 17, dihydrostreptomycin 18, erythromycin 16, carbomycin 1, polymyxin B 19, terramycin 15, tetracycline 15, triple sulpha 10, and furadantin 10. It is evident that a considerable range of antibiotics can act against these strains. There are few reports for comparison. Béguet (2) studied the changes in resistance of subcultures from a single strain. Györffy and Kállay (14) and Seligmann and Wassermann (30) studied the development of resistance to streptomycin. Weil (34) and Weinberg (35) studied inhibition of pigment formation by several antibiotics. Mollaret and Chamfeuil (22) also observed the ineffectiveness of penicillin, carbomycin, and bacitracin, as contrasted to the general effectiveness of chloramphenicol, aureomycin, terramycin, and polymyxin B. Erythromycin and tetracycline were usually active against our strains but not against theirs.

Differences between Patterns

Numerous differences between the characteristics of patterns 1 and 2 suggest that these represent 2 species-like divisions of the cultures. Differences occur in the following tests: adonitol, arabinose, dextrin, gas from glucose, lactose, lactose slant, melezitose, melibiose, methylglucoside, raffinose, xylose, and gelatin, a total of 12 features. One strain isolated by Mollaret and Chamfeuil (22) from meat very probably corresponds to pattern 2. Their strain was aerogenic, fermented lactose, arabinose, and xylose, and pigment production by this strain was better than by their other strains resembling pattern 1, especially at lower temperatures. Of the 27 carbohydrates employed, 15 were attacked by strains of pattern 1 and 23 by strains of pattern 2. A chi-square test with Yates' correction shows that the difference is significant at the 5% level. Of special practical significance is the fermentation reaction in lactose, in which the positive reaction of strains of pattern 2 could give the colonies the appearance of the coliform group when growing on

PLATE I

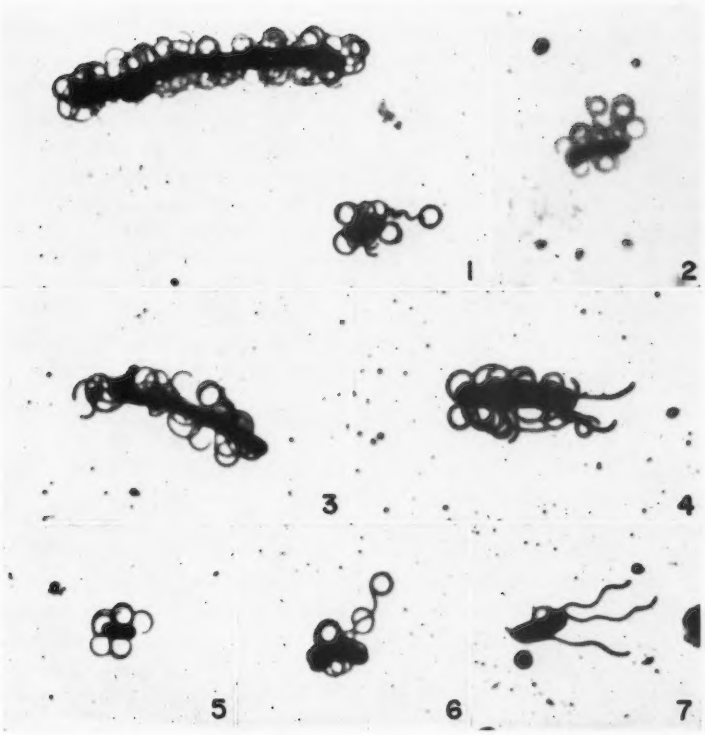

FIG. 1. *Serratia marcescens* NCTC 2302. The upper figure shows a filament with the coiled peritrichous flagella typical of *Serratia*. The lower figure illustrates what is apparently a single individual with coiled flagella and one flagellum which is curly in its proximal part and ends in a coil.
FIG. 2. *S. marcescens* 2690. Typical coiled peritrichous flagella.
FIG. 3. *S. plymuthicum* ATCC 183. Short filament with coiled peritrichous flagella.
FIG. 4. *S. kiliensis* ATCC 992. Short filament with coiled peritrichous flagella.
FIG. 5. *S. anolium* NRRL B-1700. Single individual with coiled peritrichous flagella.
FIGS. 6 and 7. *S. indica* NCTC 4002. Fig. 6 shows coiled flagella typical of *Serratia*. Fig. 7 shows apparently unusual variant with normal peritrichous flagella.

lactose agars. This key difference, useful for rapid preliminary identification, is supported by the other 11 differences noted above. Occasionally a strain of either pattern might be found in which one of the differences failed to manifest itself. Motility, for example, was lacking in certain strains which otherwise belonged to pattern 1. The gelatin test was negative with a number of strains of pattern 1, although these were stock cultures of some age and records of fresh isolations show that gelatin is probably always attacked by cultures of pattern 1 recently isolated from the wild state.

A few other peculiarities of the bacteriological reactions may be mentioned. *Serratia* strains exhibit considerable tendency toward reversion of pH in the upper strata of fermentation media. This source of confusion was somewhat decreased by incubating at 30° C. Some strains grown in tryptone broth produced so much pigment that the color showed in the medium and was extracted into the isoamyl alcohol layer in the Kovacs method for indole. Pigment was not found in 6 of the 17 strains of pattern 1 and was not brought out by any of several methods. The pigment of strains of pattern 2 was intensified by cold storage. Pigmentation may be a rather poor cardinal characteristic of strains of *Serratia*, at least of pattern 1.

Related Bacteria

The formation of acid in maltose, mannitol, salicin, and sucrose, and the lack of acidity in lactose and xylose, seems to be a device useful for the recognition of strains of pattern 1. Other groups in the family Enterobacteriaceae do not have this combination of actions on the specified carbohydrates. The imvic, gelatin, and sulphide reactions tend to relate *Serratia* to the *Cloaca* group and its neighbors, such as *Klebsiella*. It is possible that cultures of *Serratia* isolated from clinical materials are often assigned to the paracolon group, since those slow fermenters of lactose are frequently positive in salicin and sucrose. The general pattern of reactions of *Serratia* is also suggestive of the reactions of the fermentative polar flagellates known as *Aeromonas*. It is not generally known that *Aeromonas* can grow on such media as desoxycholate agar, but Hugh (16) has isolated them from clinical material. They display several differences from *Serratia*. The flagella stain is the readiest method of distinguishing them. They also have a considerable variety of imvic reactions, whereas *Serratia* are almost uniformly $--++$ in these tests. *Aeromonas* usually produce small but definite amounts of gas from glucose. In the group of strains studied, *Aeromonas* failed to ferment one or more of the carbohydrates maltose, mannitol, salicin, and sucrose, which were consistently positive with *Serratia*.

Discussion

Many of the strains reported here came from clinical sources. There is not sufficient data to assess their pathological significance. The literature cited suggests that these strains are not harmless, but may be secondary

invaders and contribute to the severity and the prolongation of the pathological condition. The dearth of evidence of clinical significance may be a result of failure of the laboratory to recognize *Serratia* when it is present. Dependence on pigmentation will apparently lead to passing over of many, perhaps half, of the strains isolated. The remarkable coiled form of the flagella would be a more reliable cardinal characteristic. The findings suggest that this is a generic characteristic. They also suggest that the pattern 1 strains, which conform more to the classical concept of the species *S. marcescens*, are the ones to be expected from clinical sources. Even in the absence of the flagella stain, these unpigmented cultures may be recognized by the following set of characteristics: anaerogenic, sulphide negative, and producing acid in maltose, mannitol, salicin, and sucrose while negative in xylose.

References

1. Aronson, J. D. and Alderman, I. The occurrence and bacteriological characteristics of S. marcescens from a case of meningitis. J. Bacteriol. **46**, 218 (1943).
2. Béguet, M. Action de quelques antibiotiques sur divers types de B. prodigiosus issus de la meme souche. Ann. inst. Pasteur, **85**, 394–396 (1953).
3. Böhlck, I. Über eine Prodigiosus-Infektion. Ärztl. Wochschr. **11**, 19–21 (1956).
4. Breed, R. S. (Ed.) Bergey's manual of determinative bacteriology. 7th ed. The Williams & Wilkins Co., Baltimore, Md. 1957.
5. Bruner, D. W. The preparation and use of a polyvalent Salmonella antiserum. Cornell Vet. **47**, 491–496 (1957).
6. Capponi, M., Sureau, P., Le Minor, L., Drean, D., and Vo-Thann. Contribution a l'étude des salmonelles du Centre-Vietnam. Bull. soc. pathol. exotique, **49**, 796–801 (1956).
7. Cutin, M. and Sapuppo, R. Otite media cronica supurada, por Bacillus prodigiosus. Rev. paul. med. **39**, 358–359 (1951).
8. Deom, J. and Mortelmans, J. Étude d'une souche pathogène de Serratia marcescens. Rev. immunol. **17**, 394–398 (1953).
9. Duran-Reynals, F. and Clausen, A. J. A contagious tumor-like condition in the lizard (*Anolis equestris*) as induced by a new bacterial species, *Serratia anolium* (sp. n.). J. Bacteriol. **33**, 369–380 (1937).
10. Editorial. Infection due to chromobacteria. J. Am. Med. Assoc. **148**, 56 (1952).
11. Editorial. Chromobacterial infections. Lancet, **2**, 617–618 (1952).
12. Gale, D. and Lord, J. D. Overgrowth of Serratia marcescens in respiratory tract, simulating hemoptysis; report of a case. J. Am. Med. Assoc. **164**, 1328–1330 (1957).
13. Gurevitch, J. and Weber, D. A strain of Serratia isolated from urine. Am. J. Clin. Pathol. **20**, 48–49 (1950).
14. Györffy, B. and Kállay, I. Streptomycin resistance of Serratia marcescens. Acta Microbiol. Acad. Sci. Hung. **4**, 309–331 (1957).
15. Hawe, A. J. and Hughes, M. H. Bacterial endocarditis due to Chromobacterium prodigiosum; report of a case. Brit. Med. J. **1**, 968–970 (1954).
16. Hugh, R. Personal communication (1958).
17. Khinchuk, A. G. Bacillus prodigiosus isolated in pyelitis. Rev. Microb., epidémiol. et parasitol. **9**, 112–113 (1930).
18. Klein, E. On an infection of food-stuffs by Bacillus prodigiosus. J. Pathol. Bacteriol. **2**, 217–218 (1894).
19. Kwapinski, J. Pathogenic properties of Serratia. Polski tygod. lek. **10**, 891–893 (1955).
20. Levy, J. Meningitis due to Serratia marcescens. Harefuah, **42**, 87–88 (1952).
21. Lo Presti, J. M., Kaufman, P., Olshaker, B., Ross, S., and Stevens, S. The use of chloramphenicol ("Chloromycetin") in the treatment of infections of the urinary tract in childhood; special report. Clin. Proc. Child. Hosp., Wash. **6**, 177–181 (1950).
22. Mollaret, L. and Chamfeuil, R. Apparition du genre Serratia en pathologie humaine: étude microbiologique de 15 souches. Ann. inst. Pasteur, **94**, 643–653 (1958).
23. Mortelmans, J. Personal communication (1955).
24. Paine, T. F. Illness in man following inhalation of Serratia marcescens. J. Infectious Diseases, **79**, 226–232 (1946).
25. Patterson, R. H., Jr., Banister, G. B., and Knight, V. Chromobacterial infection in man. A.M.A. Arch. Internal Med. **90**, 79–86 (1952).

26. RABINOWITZ, K. and SCHIFFRIN, R. A strain of Serratia marcescens isolated from a case of meningitis in a child. Harefuah, **42**, 88–90 (1952).
27. RABINOWITZ, K. and SCHIFFRIN, R. A ward-contamination by Serratia marcescens. Acta Med. Orient. **11**, 181–184 (1952).
28. RISKÓ, T., NIKODEMUSZ, I., and TOMKA, I. Pathogenic effect of the Bacillus prodigiosus (Serratia marcescens). Orvosi Hetilap, **93**, 673–674 (1952).
29. ROBINSON, W. and WOOLLEY, P. B. Pseudohaemoptysis due to Chromobacterium prodigiosum. Lancet, **1**, 819 (1957).
30. SELIGMANN, E. and WASSERMANN, M. Induced resistance to streptomycin. J. Immunol. **57**, 351–360 (1947).
31. SIGTENHORST, M. L. Techniques for the isolation and identification of enteric bacilli. Am. J. Med. Technol. **20**, 79–89 (1954).
32. VERNON, R. G. and HEPLER, O. E. Chromogenic bacteria with a case report of a fatal infection caused by Serratia marcescens. Quart. Bull. Northwestern Univ. Med. School, **28**, 366–372 (1954).
33. WAISMAN, H. A. and STONE, W. H. The presence of Serratia marcescens as the predominating organism in the intestinal tract of the newborn; the occurrence of the "red diaper syndrome". Pediatrics, **21**, 8–12 (1958).
34. WEIL, A. J. Inhibition of pigment formation of Serratia marcescens by chloramphenical, aureomycin and terramycin. Proc. Soc. Exp. Biol. Med. **79**, 539–540 (1952).
35. WEINBERG, E. D. The influence of antibiotics and amino acids on the production of pigment by Serratia. J. Bacteriol. **62**, 785–792 (1951).
36. WHEAT, R. P., ZUCKERMAN, A., and RANTZ, L. A. Infection due to chromobacteria; report of eleven cases. A.M.A. Arch. Internal Med. **88**, 461–466 (1951).

PURIFICATION AND PROPERTIES OF THE 2-KETOGLUCONOKINASE OF LEUCONOSTOC MESENTEROIDES[1]

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Abstract

A specific and adaptive 2-keto-D-gluconokinase has been isolated from cells of *Leuconostoc mesenteroides* grown on 2-keto-D-gluconate. The enzyme, purified 138-fold, has an optimum pH of 7.7, is most stable between pH 6.0 and 8.0, and is quickly inactivated at temperatures above 40° C. Magnesium chloride is required for activity. ATP, ITP, and GTP served as phosphate donors but ADP and UTP were inactive. Whereas EDTA and glycine stabilized the enzyme, *p*-chloromercuribenzoate, hydroxylamine, copper acetate, mercury acetate, and sodium fluoride were inhibitory. The product of the enzymatic reaction was isolated and characterized as 2-keto-6-phospho-D-gluconate.

Introduction

De Ley and Vandamme (12) surveyed a number of microorganisms for ability to grow on 2KG[3] and for the production of 2-ketogluconokinase. Positive results were obtained with bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Escherichia*, *Aerobacter*, *Paracolobactrum*, *Serratia*, *Erwinia*, and *Bacillus*. None of the other species of bacteria, fungi, or yeasts tested showed any 2-ketogluconokinase activity, although some were capable of using 2KG as the sole carbon source. Evidence has been reported for the occurrence of this enzyme in *Corynebacterium creatinovorans* (20), *Acetobacter suboxydans* (16), *Acetobacter melanogenus* (24), and *Aerobacter aerogenes* (18). The phosphorylation of 2KG has been studied in detail in *Aerobacter cloacae* (10), *Pseudomonas fluorescens* (29), and *Aerobacter aerogenes* (19).

Previous work in this laboratory has indicated that 2KG may be utilized as a carbon source by *L. mesenteroides* (3) and that the final products are identical with those from other carbohydrates, i.e., equimolecular amounts of carbon dioxide, lactate, and acetate (5). It has been suggested (4) that the first step in the degradation of 2KG is the formation of the phosphate ester. The present study supports this view since a specific 2-ketogluconokinase has been isolated from *L. mesenteroides* and its reaction product characterized as 2K6PG.

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[2]National Research Council of Canada Postdoctorate Fellow, 1957–1959.
[3]The following abbreviations are used: 2KG, 2-keto-D-gluconate; 2K6PG, 2-keto-6-phospho-D-gluconate; 6PG, 6-phospho-D-gluconate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; UTP, uridine triphosphate; GSH, reduced sodium glutathione; EDTA, sodium ethylenediaminotetraacetate; TRIS, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; DEAE-cellulose, diethylaminoethylcellulose.

Materials and Methods

Bacteriological

Leuconostoc mesenteroides, PRL 33, was maintained and grown as previously described (1, 3). Large batches of cells were obtained by growing the organism in 7 liters of medium in glass bottles. Three to four days before inoculation a suspension of the organism in the growth medium, stored at −20° C, was quickly thawed and incubated at 37° C for 18 to 24 hours. The culture was then serially transferred to tubes of freshly prepared medium until vigorous growth was obtained (2 to 3 days). Twenty milliliters of an 18-hour-old culture was used to inoculate the 7 liters of medium. After growth at 30° C for 18 hours, the cells were harvested by centrifugation, washed with distilled water, and suspended in 100 ml of a neutral solution of 0.002 *M* cysteine hydrochloride and 0.001 *M* EDTA.

The suspension of cells was treated in a 10 kc Raytheon sonic oscillator at 0 to 8° C for 25 minutes and the crude extract centrifuged (20,000×*g* for 20 minutes). If necessary, the unbroken cells were resuspended and the treatment repeated. The cells or the crude extracts were always stored at −20° C.

Materials

The materials used in this investigation were the same as those previously described (3).

Dr. V. F. Pfeifer of the Northern Utilization Research and Development Division, Peoria, Illinois, generously donated a sample of calcium 2-ketogluconate. Additional supplies were obtained by oxidizing glucose with *Pseudomonas fragi*, NRRL B-25 (supplied by Dr. Pfeifer), in 15 liters of medium in a stainless steel fermentor. The oxidation, in a medium consisting of 10% cerelose, 0.2% yeast extract, 0.2% N-Z Tone, 0.025% $MgSO_4.7H_2O$, 0.06% KH_2PO_4, 0.2% urea, and 2.7% calcium carbonate that was aerated at the rate of 10 liters of air per minute, was complete in 48 hours with a yield of 80%. The disappearance of glucose was followed by the method of Willstäter and Schudel (2) and the production of 2KG by 2-ketogluconokinase. Calcium 2KG was recovered by the procedure of Pfeifer *et al.* (33).

5-Keto-D-gluconate was a gift of Prof. R. Ciferri, University of Pavia, Pavia, Italy. ITP was purchased from Nutritional Biochemical Corporation, ADP and UTP from Pabst Laboratories, ATP and GTP from Sigma Chemical Company, the acid phosphatase from Worthington Biochemical Corporation, and DEAE-cellulose from Brown Company.

Methods

Kinase activity was measured at 30° C by the manometric procedure of Colowick and Kalckar (7) with appropriate controls for adenosine triphosphatase activity. The reaction mixture (volume = 1 ml) contained 10 μM EDTA (pH 7.7), 60 μM $NaHCO_3$ (pH 7.7), 20 μM ATP (pH 7.7), 2-ketogluconokinase (8 units), 30 μM $MgCl_2$ (pH 7.0), and 20 μM substrate (pH 7.0), added in that order. The reaction was initiated by tipping in the substrate from the side arm and the volume of carbon dioxide released between the 5th

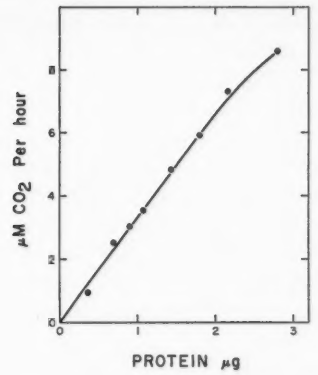

FIG. 1. Effect of enzyme concentration on the reaction rate. The reaction mixture contained 20 μM of 2KG, 30 μM of $MgCl_2$, 20 μM of ATP, 10 μM of EDTA, 45 μM of $NaHCO_3$, and 2-ketogluconokinase as shown.

and 20th minute used to calculate the units of activity. One unit was defined as the amount of enzyme required to phosphorylate 1 μM of 2KG per hour. The velocity of this reaction was essentially proportional to the concentration of enzyme up to 16 units (Fig. 1).

Growth of the organism was determined photometrically on a Coleman Junior spectrophotometer at 620 mμ against a blank of uninoculated medium.

Proteins were determined according to the procedure of Lowry *et al.* (27) or by the spectrophotometric method of Kalckar (23); reducing sugar was determined by the procedures of Somogyi (36) and Nelson (30); and inorganic phosphate by the method of Fiske and Subbarow (17).

Ascending paper chromatograms were developed at 2° C on Whatman No. 54 with methanol–ammonia–water (60:10:30; v/v) or acetone – 25% TCA (40:10; v/v). The sugars were detected by means of the *o*-phenylenediamine reagent (25), and, occasionally, by using ammonium molybdate (21), bromcresol green (28), or *p*-anisidine sprays (22).

2K6PG was prepared by mixing 1 mM of EDTA, 2 mM of ATP, 3 mM of $MgCl_2$, 1.5 mM of 2KG, and 5000 units of 2-ketogluconokinase (final volume 25 ml, pH 7.5). The mixture was held at room temperature and the pH maintained at 7.2 to 7.5 by periodic addition of 1 *N* NaOH. Phosphorylation was complete in 4 hours.

2K6PG was isolated by the excellent procedure developed by Frampton (19). The solution containing 2K6PG was applied to a Dowex-1 formate column (22×1.2 cm) (35), and the column washed with 50 ml of water. The 2K6PG was recovered by gradient elution with 4 *N* formic acid and ammonium formate as described by Frampton (19). The elution rate was 2 ml per minute and 10-ml fractions were collected. A single peak with reducing activity (30, 36) was obtained between tubes numbered 84 and 93. These eluates were pooled and the pH adjusted to 6.0 with ammonium hydroxide and then

lyophilized. The light-yellow oil so obtained was dissolved in water, deionized with Dowex-50 (H^+ form), adjusted to pH 6.0 with dilute potassium hydroxide, and relyophilized. The product, recovered as a white powder, represented 985 μM of 2K6PG (calculated as anhydrous di-K salt) with a yield of 65% of the theoretical.

At times considerable difficulty was encountered in obtaining 2K6PG in crystalline form since the ester apparently decomposes rapidly giving rise to a hygroscopic yellow-brown gum. This difficulty was partially overcome by a treatment with acid-washed charcoal which removed most of the colored matter. Even so, the preparations tended to decompose at room temperature and in a desiccator. Storage at $-15°$ C over calcium chloride was only partially beneficial.

2K6PG may also be recovered from the eluate by extracting the formic acid with ether, converting 2K6PG to the free acid with Dowex-50 (H^+ form), neutralizing with dilute potassium hydroxide, and precipitating the potassium salt with 4 volumes of ethanol.

Experimental

Distribution of the Kinase Activity

Cells grown on five different substrates (2KG, glucose, gluconate, fructose, and xylose) were treated in the sonic oscillator and the ability of the crude extracts to phosphorylate various carbohydrates determined (Table I). 2-Ketogluconokinase activity was evident only in cells grown on 2KG. The xylulokinase of *L. mesenteroides* is likewise an inducible enzyme, present only when the organism is grown on xylose (39).

TABLE I

Phosphorylation of various substrates (specific activity*)

Cells grown on	2KG	Glucose	Gluconate	Fructose	Mannose	Xylose	Xylulose
2KG	25	0	17	45	31	0	0
Glucose	0	5	7	11	11	0	0
Gluconate	0	1	13	3	1	0	0
Fructose	0	0	13	16	14	0	0
Xylose	0	0	22	34	29	8	18

*Units per milligram of protein.

The phosphorylation of gluconate, fructose, and mannose appears to be catalyzed by constitutive enzymes, present regardless of the substrate on which the organism is grown. Evidence obtained during the fractionation of 2-ketogluconokinase suggests there are at least three kinases present, one specific for 2KG, one specific for gluconate, and a third for fructose and mannose.

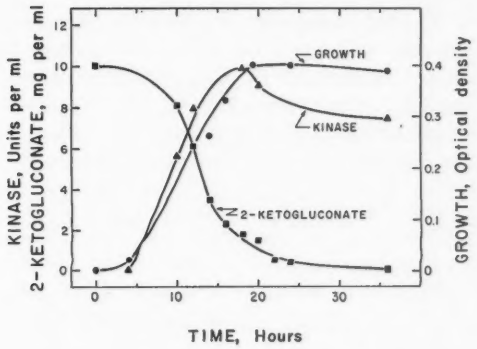

FIG. 2. The relation between growth, consumption of 2-ketogluconate, and production of 2-ketogluconokinase. Medium: 10 g 2KG, 10 g yeast extract, 10 g Bacto tryptone, 2.5 g KH_2PO_4, 2.5 g Na_2HPO_4, 20 ml inorganic salts (0.8% $MgSO_4.7H_2O$, 0.04% $FeSO_4.7H_2O$, 0.04% NaCl, 0.19% $MnCl_2.4H_2O$, and 0.5% concentrated HCl), and 980 ml distilled water. The solutions containing 2KG, the phosphate buffer, and the inorganic salts were sterilized separately and aseptically added to the yeast extract and tryptone solution before inoculation.

Production of 2-Ketogluconokinase

Growth, utilization of 2-ketogluconate, and formation of 2-ketogluconokinase by *L. mesenteroides* in 7 liters of medium was determined by aseptically withdrawing samples at suitable intervals for analysis. The results depicted in Fig. 2 show that growth and production of 2-ketogluconokinase were concomitant, the maxima being attained 18 hours after inoculation. This suggests a correlation between growth and formation of 2-ketogluconokinase.

After 18 hours, the cell population remained practically constant for another 16 hours, while the amount of 2-ketogluconokinase decreased. Therefore, cells obtained by growth on 2KG were harvested 18 hours after inoculation. When grown on other substrates the cells were harvested 12–18 hours after inoculation.

Purification of 2-Ketogluconokinase

Unless otherwise stated, all operations were performed at 0 to 5° C. Precipitates were separated by centrifugation at $11,100 \times g$ for 10 minutes in a Lourdes centrifuge or at $20,000 \times g$ in a Servall SS1 centrifuge. EDTA buffer (1×10^{-3} *M*, pH 7.6) was used to dissolve precipitates and for dialysis.

Magnesium Sulphate Precipitation

One hundred and seventy-two grams of $MgSO_4.7H_2O$ was added to 240 ml of cell-free extract (pH 7.2, 14.8 mg of protein per ml) with mechanical stirring at room temperature. On addition of the salt, the pH dropped to 5.2 and the precipitate that formed was removed by centrifugation. The supernatant solution was adjusted to pH 7.4 by dropwise addition of 1.0 *N* sodium hydroxide and allowed to stand at room temperature for 1 hour. A second precipitate was removed by centrifugation and the supernatant dialyzed overnight

against flowing 0.001 *M* EDTA buffer. High osmotic pressures developed during dialysis and sometimes it was necessary to change bags to avoid bursting. The white, flocculent precipitate that formed was centrifuged off and the pH of the supernatant adjusted from 5.2 to 5.5. (Magnesium sulphate, 1180 ml; 1.0 mg of protein per ml.) The ratio of optical densities at 280 and 260 mμ (E 280/E 260) which was 0.59 for the crude extract increased to 0.71.

Adsorption of Foreign Proteins with Charcoal

For each milliliter of the supernatant solution, 10 mg of acid-washed Norit-A (8) was added and the solution stirred for 5 minutes. The charcoal was removed by filtration through Whatman No. 2 filter paper. The E 280/E 260 increased to 0.75. (Charcoal supernatant, 1400 ml; 0.70 mg of protein per ml.)

Adsorption of the Enzyme on Calcium Phosphate Gel

The charcoal supernatant was treated with 140 ml of a calcium phosphate gel (30 mg per ml) in 0.001 *M* EDTA buffer. The mixture was stirred for 5 minutes, centrifuged, and the supernatant discarded. The enzyme was recovered from the gel by eluting twice with 100 ml of 0.25 *M* potassium phosphate buffer in 0.001 *M* EDTA, pH 7.5. (Calcium phosphate eluate, 250 ml; 2.7 mg of protein per ml.)

Removal of Nucleoproteins

The eluate was diluted 1:1 with 0.001 *M* EDTA and treated with 20 ml of a 1% solution of protamine sulphate, pH 5.5. The solution was stirred for 10 minutes and the precipitate removed by centrifugation. The ratio E 280/E 260 of the supernatant increased to 1.1. (Protamine supernatant, 560 ml; 1.2 mg of protein per ml.)

Ammonium Sulphate Fractionation I

The protamine supernatant was treated with 194 g of solid ammonium sulphate (57% saturation) and sufficient ammonium hydroxide to maintain a neutral pH. The precipitate was discarded and an additional 95 g of ammonium sulphate (80% saturation) was added to the supernatant. The precipitate was collected by centrifugation and dissolved in EDTA buffer (36 ml), then adjusted to pH 7.5 and dialyzed overnight against flowing EDTA. (Ammonium sulphate I, 65 ml; 8.1 mg of protein per ml.)

Chromatography on DEAE-Cellulose Column

Nine grams of commercial DEAE-cellulose were suspended in 100 ml of EDTA buffer and poured into a glass column (diameter 1.3 cm). The cellulose was packed by applying a pressure of approximately 4 lb/in^2 (height 16 cm). The column was washed with 50 ml of EDTA buffer and the dialyzed ammonium sulphate fraction (pH 6.0) applied. When the enzyme was adsorbed, the column was washed with 50 ml of EDTA buffer and then with 65 ml of 0.06 *M* potassium phosphate buffer in 0.001 *M* EDTA, pH 6.0. This was followed by a gradient elution using 100 ml of 0.06 *M* phosphate buffer in the

mixing chamber and 300 ml of 0.12 *M* phosphate buffer in the reservoir, both in 0.005 *M* EDTA and at pH 6.7. The elution rate was approximately 2.5 ml per minute and the fractions containing kinase with a specific activity of 1000 or more were pooled and the pH adjusted to 7.5. (Cellulose column eluate, 90 ml; 0.4 mg of protein per ml.) This step could be increased or decreased in size as long as the gradient elution was not begun until the pH of the eluate obtained by applying 0.06 *M* potassium phosphate in 0.001 *M* EDTA buffer (pH 6.0) had dropped to pH 7.0–7.5. A typical elution pattern is depicted in Fig. 3.

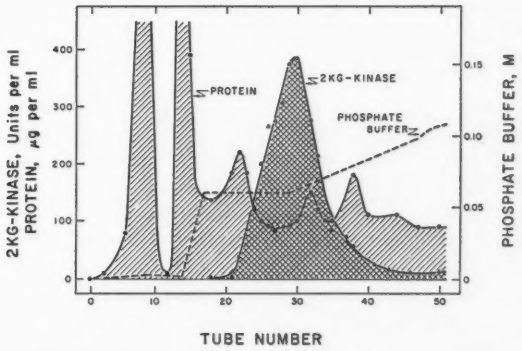

FIG. 3. Chromatographic separation of 2-ketogluconokinase on DEAE-cellulose column. Three grams of DEAE were suspended in 0.001 *M* EDTA (pH 7.6) and poured in the column (final dimensions: 1.1 cm × 6.0 cm). The enzyme (24 ml, 4.8 mg of protein and 850 units per ml, pH 6.0) was applied on the column and, after adsorption, washed with 50 ml of 0.001 *M* EDTA (pH 7.6). The washing with 0.06 *M* KH_2PO_4 and the gradient elution were carried out as described in the text. 2-Ketogluconokinase was recovered from tubes numbered 25 to 35. A preparation with fructokinase activity was eluted in tubes 37–40, corresponding to the last protein peak.

Ammonium Sulphate Fractionation II

To the combined eluates was added 25 g of solid ammonium sulphate and the precipitate discarded. Ammonium sulphate (32.1 g; 48–60% saturation) was added to the supernatant and the mixture allowed to stand for 15 minutes. The precipitate was recovered by centrifugation, dissolved in EDTA buffer, and adjusted to pH 7.5. (Ammonium sulphate II, 12 ml; 0.6 mg of protein per ml.)

The 138-fold purified preparation was free of other kinases and ATP-ase (Table II).

Stability of 2-Ketogluconokinase

Cells stored at −20° C retained their original activity for at least 3 weeks and after 28 weeks' storage 82% of the activity remained. Storage of crude extracts for 12 weeks at −20° C did not result in any loss of activity. A 138-fold purified preparation (ammonium sulphate II) after 1 week at −20° C retained its original activity; however, after 12 weeks the activity was 41% of the original.

TABLE II

Purification of 2-ketogluconokinase

Treatment	Total activity, units $\times 10^{-3}$*	Specific activity, units/mg protein	Purification, fold	Yield, %
Crude extract	120	34	0	100
Magnesium sulphate	110	93	2.7	92
Charcoal	110	98	2.9	92
Calcium phosphate gel	117	142	4	98
Protamine sulphate	120	178	5	100
Ammonium sulphate I (57–80%)	115	262	8	96
Dialysis	98	186	6	85
DEAE-cellulose column	54	2,005	60	45
Ammonium sulphate II (48–60%)	32	4,700	138	27

*1 unit = 1 μM of 2KG phosphorylated per hour.

Stability of the enzyme was not improved by increasing or decreasing the concentration of EDTA, adding magnesium chloride or 2KG. Ammonium sulphate (approximately 5%) appears to have a stabilizing effect.

The enzyme is stable at 30° C for 30 minutes over a rather wide pH range (Fig. 4). When held at 40° C for half an hour inactivation is less rapid in the presence of glycine. Above 40° C the enzyme, even at neutrality, loses activity very quickly. When held at 45° C for 10 minutes, 64% of the activity is destroyed, and at 50° C complete inactivation occurs.

Effect of pH

In bicarbonate buffer maximum phosphorylation occurs between pH 7.7 and 8.0 (Fig. 5). Above pH 8.0 the rate declines fairly rapidly while no accurate measurements can be obtained below pH 7.2 (37).

Activation and Inhibition

Preparations purified more than 100-fold were stimulated by EDTA up to a concentration of 2.5×10^{-6} *M* while higher concentrations were inhibitory (Fig. 6). Low concentrations of GSH resulted in slight activation while higher concentrations (1×10^{-5} *M*) resulted in inhibition. Cysteine and sodium thioglycolate inhibited even in low concentrations (5×10^{-7} *M*). Sodium ascorbate between 5×10^{-7} and 1×10^{-5} *M* was innocuous. Preparations purified 20- to 50-fold were not affected by EDTA, GSH, and other thiols. The requirement for magnesium was evident even in the crude extracts since no activity could be detected in its absence.

Of the inhibitors tested (Table III), *p*-chloromercuribenzoate was the most effective. 2K6PG partially inhibited the phosphorylation of 2KG. In assays containing 20 μM of 2KG, the addition of two levels of 2K6PG (8.4 and 16.8 μM) resulted in an inhibition of 8% and 11%, respectively.

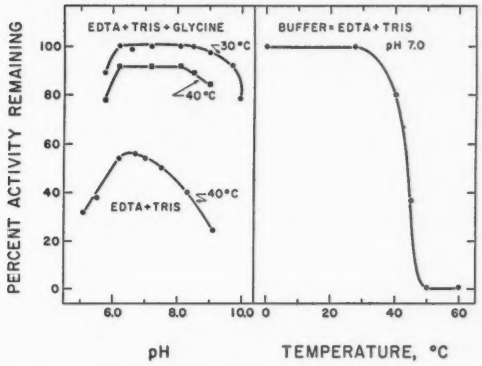

FIG. 4. Stability of 2-ketogluconokinase as a function of pH and temperature.
Left: 0.1 Milliliters of enzyme solution (200 units) was added to 0.4 ml of 0.1 *M* TRIS + EDTA + glycine buffer (or TRIS + EDTA buffer) at the desired pH and held in a water bath for 30 minutes. The solutions were quickly cooled in an ice–ethanol bath and 0.1 ml transferred to 0.9 ml of 0.1 *M* EDTA + TRIS buffer at pH 7.0. The pH was determined on the remainder of the heat-treated preparation. The controls were held at 0° C.
Right: 0.1 Milliliters of enzyme solution (200 units) at pH 7.0 was diluted with 0.1 ml of glass distilled water and held for 5 minutes in water bath at the desired temperature. The samples were then quickly cooled by immersion in an ice–ethanol bath and assayed.

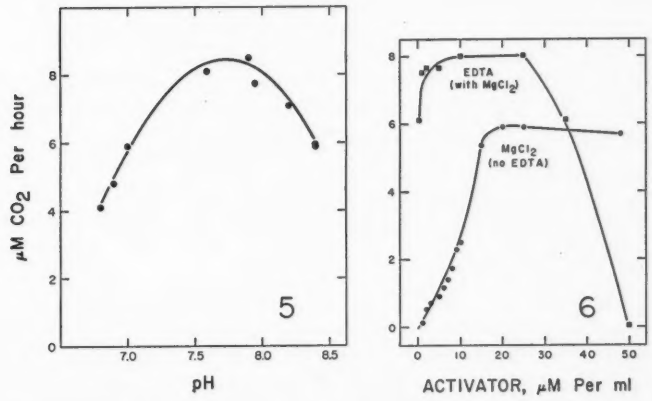

FIG. 5. Activity of 2-ketogluconokinase as a function of pH. Conditions as in Fig. 1 except that the concentration of bicarbonate was varied to give the desired pH (37), and 8 units of 2-ketogluconokinase were used. The pH was determined at the end of the reaction with a glass electrode.
FIG. 6. Effect of EDTA and $MgCl_2$ upon the activity of 2-ketogluconokinase. The reaction mixture was the same as in Fig. 1 except that 60 μM of $NaHCO_3$ and 8 units of 2-ketogluconokinase were used and the $MgCl_2$ and EDTA concentrations varied as shown. EDTA was omitted from the flasks in which $MgCl_2$ was varied.

TABLE III

Inhibition of 2-ketogluconokinase

Inhibitor	Concentration, μM/ml	Inhibition, %
Sodium fluoride	25	0
	100	55
Hydroxylamine	100	0
	300	66
Potassium cyanide	300	0
p-Chloromercuribenzoate	10	100
Copper acetate	100	100
Mercury acetate	100	100

NOTE: 205 Units of 2-ketogluconokinase (0.1 ml) were added to standard quantities of inhibitor solutions to give the concentration shown above and held in an ice bath for 10 minutes. Aliquots were withdrawn for assay under standard conditions; controls taking into account the dilutions were run using glass-distilled water dilutions of the enzyme solution.

Nucleotide Specificity

At the level of 20 μM per ml, ADP and UTP were inactive as phosphate donors while ITP and GTP could partially replace ATP. The rate of phosphorylation with ITP was 80% and that of GTP 45% of that for ATP. In the presence of ADP (10 μM per ml), however, neither ITP nor GTP (20 μM per ml) could be utilized. ADP was only slightly inhibitory in reactions containing ATP. Thus when the ratios ADP/ATP were 1:5 and 1:2 the amount of inhibition was 4% and 15% respectively.

Substrate Specificity

The 138-fold purified preparation phosphorylated only 2KG. With 20 units of 2-ketogluconokinase no reaction was obtained with the following substrates (20 μM per ml): 5-keto-D-gluconate, D-glucose, D-fructose, D-mannose, D-gluconate, 2-deoxy-D-glucose, D-xylose, D-ribose, D-arabinose, D-sorbose, D-lactose, D-galactose, D-xylulose, D-ribulose, D-sedoheptulose, D-arabitol, D-adonitol, D-mannitol, D-sorbitol, *i*-erythritol, D-glucosamine, and α-heptonic acid.

Stoichiometry

Under conditions of the manometric assay and with excess of 2-ketogluconokinase (56 units), the addition of three levels of 2KG (1.0, 2.0, and 3.0 μM) resulted in carbon dioxide evolution, which, upon completion in 30 minutes, was respectively 97.5, 88.5, and 85.5% of that expected for one phosphorylation per μM of 2KG.

Estimation of the Michaelis constant (K_s) would indicate that the affinity of the enzyme for 2KG is twice as much as that for ATP (Fig. 7). An estimation of the affinity of the enzyme for magnesium chloride could not be obtained (Fig. 6).

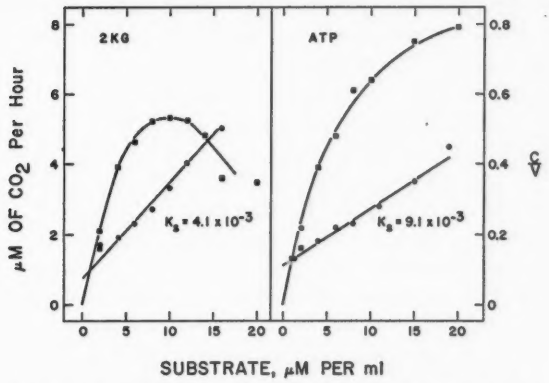

FIG. 7. Effect of the concentration of ATP and 2KG on the rate of phosphorylation. The reaction mixture was the same as in Fig. 6 except that 30 μM of $MgCl_2$ and 10 μM of EDTA were used and the concentrations of 2KG and ATP were varied as shown.

Identification of the Phosphorylation Product

The properties of the phosphorylated product are summarized in Table IV. By paper chromatography, 2K6PG is easily distinguished from the unphosphorylated sugar by the different R_f values and different colors obtained with the *o*-phenylenediamine spray (25). While 2KG gives a yellow-green color with yellow fluorescence under ultraviolet light, 2K6PG gives a violet non-fluorescent spot. The violet non-fluorescent spot is specific for 2K6PG

TABLE IV

Properties of the phosphorylation product

Test	Kinase product	2KG	Dephospho compound
Derivative: quinoxaline (absorbancy)	Maximum, 335 mμ	Maximum, 335 mμ	
Optical rotation (degrees)	$[\alpha]_D^{24}$ +3.3°	$[\alpha]_D^0$ −69.95° (31)	
Phosphate: 2KG	1		
1 *N* H_2SO_4, 94° C, 150 minutes (P/mole)	0.80		
Chromatography (R_f): MeOH–NH_4OH–H_2O (60:10:30)	0.36	0.60	0.60
Acetone–25% TCA (40:10)	0.79	0.66	0.66
Spray reactions: *o*-Phenylenediamine	Violet non-fluorescent	Yellow-green fluorescent	Yellow-green fluorescent
Bromcresol green	Yellow	Yellow	Yellow
p-Anisidine	Yellow-brown	Yellow-brown	Yellow-brown
Ammonium molybdate	Blue	Colorless	Colorless

(10, 11, 29). All three compounds tested (2KG, dephosphorylated product, and 2K6PG) gave positive results on treatment with bromcresol green and *p*-anisidine, while only 2K6PG gave a positive test for organic phosphate on spraying with ammonium molybdate reagent.

The phosphate group was removed enzymatically from 2K6PG; to 2.15 μM of 2K6PG in 0.05 ml was added 1.0 ml of 0.1 *M* TRIS + EDTA buffer at pH 5.5, 0.5 ml of 0.3 *M* magnesium chloride solution, and 0.45 ml of water. Ten milligrams of acid phosphatase was added and the solution incubated at 37° C for 2 hours. The control contained 0.45 ml of 0.25 *N* sulphuric acid in place of the water and was held at 0° C for the same period of time. A total of 2.17 μM of inorganic phosphate were released compared with 2.15 μM of phosphorus calculated for 1 μM of phosphorus per μM of phosphorylated sugar. Eighty per cent of the theoretical phosphorus was hydrolyzed after $2\frac{1}{2}$ hours at 94° C in 1.0 *N* sulphuric acid (Fig. 8).

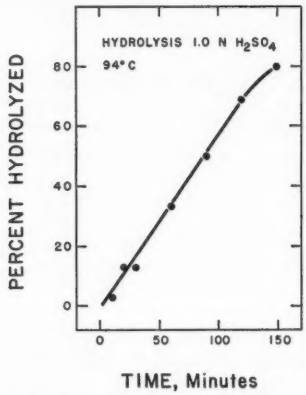

FIG. 8. The hydrolysis of 2K6PG by acid. Seven micromoles of phosphate ester (1.0 ml) were mixed with 1.0 ml of 2 *N* H_2SO_4 and held in a water bath at 94° C. 0.2-Milliliter aliquots were withdrawn at intervals and added to 0.8 ml of a 0.1 *M* TRIS + EDTA buffer at pH 5.5 in an ice–ethanol bath. Inorganic phosphate was determined by the procedure of Fiske and Subbarow (17) and total phosphate by a wet ashing procedure.

The specific rotation was determined on the potassium salt in water in a Rudolph polarimeter. 2K6PG gave $[\alpha]_D^{24}$ + 3.3° (*c*, 1.50) which is quite distinct from the value reported for the unphosphorylated compound $[\alpha]_D^0$ −69.95° (*c*, 2.216, water) (31).

De Ley (10) reported that 2K6PG may also be characterized by its ultraviolet absorption spectrum which exhibits a typical peak at 260 mμ while no such peak is shown by 2KG. The absorption was attributed to the carbonyl group of 2K6PG as the compound exists, at least in part, in the open chain form. In our experiments, the peak at 260 mμ was shown only by preparations obtained by eluting the spot of 2K6PG from paper chromatograms with a procedure similar to that used by De Ley (10). The 2K6PG purified

by column chromatography did not show absorption at 260 mμ. Furthermore, the infrared spectra of all preparations did not contain a peak for a free carbonyl group (also not shown by fructose-6-phosphate). Both 2KG and 2K6PG have an absorption peak at 1600–1625 cm^{-1} which is ascribed to the carbonyl group of the carboxyl group. The preparations obtained by paper chromatography probably were contaminated by some nucleotides (ATP or ADP) present in the reaction mixture that were not completely separated from 2K6PG on the paper chromatograms. Such nucleotides could be responsible for the absorption at 260 mμ.

Discussion

When 2KG is the only carbohydrate supplied, *Leuconostoc mesenteroides* is induced to produce 2-ketogluconokinase, which, like other adaptive enzymes, is specific for 2KG. This is also true for the 2-ketogluconokinase of *Aerobacter cloacae* (9). On the other hand, 2-ketogluconokinase is apparently a constitutive enzyme in *Pseudomonas fluorescens* (29), *Acetobacter suboxydans* (16), and *A. melanogenus* (24) since glucose-grown cells of such bacteria phosphorylate 2KG. However, these bacteria normally oxidize gluconate to 2KG in the course of glucose or gluconate degradation. The presence of 2-ketogluconokinase in glucose-grown cells of *Corynebacterium creatinovorans* cannot be stated with certainty, as already reported by the same authors (20).

A specific kinase for gluconate has been reported for a number of microorganisms (6, 9, 34, 38) and in the course of fractionation we have obtained distinct fractions that phosphorylate gluconate, fructose, and mannose. The fructo-mannokinase can be partially separated by ammonium sulphate fractionation as it precipitates at 60–85% saturation. It is completely separated on the DEAE-cellulose column where it is eluted after 2-ketogluconokinase. A detailed study of such kinases, as well as hexokinase, was beyond the scope of this work, but it appears that one enzyme is responsible for the phosphorylation of glucose and another for fructose and mannose. The hexokinase content of all preparations, even in cells grown on glucose, was always low and often undetectable, while the activity on fructose and mannose was always considerable. Hexokinase has been reported to be a constitutive enzyme of *L. mesenteroides* (14) so that our findings are at present unexplainable. All preparations and fractions which phosphorylated fructose were also active on mannose. Whether this is due to a single enzyme, to two closely related enzymes, or to the presence of an enzyme that converts mannose to fructose as found in certain fructose-grown bacteria (32) cannot be stated.

The phosphorylation of 2KG to 2K6PG is the first step in the pathway of 2KG degradation. 2K6PG may then be reduced to 6PG by a specific reductase as found in other species of bacteria (13, 18, 19), or, alternatively, could undergo decarboxylation to a pentose. The second possibility would confirm the classical hypothesis of earlier investigators (15, 26).

Acknowledgment

The authors are indebted to Dr. A. S. Perlin and Dr. E. M. von Rudloff for helpful suggestions, and to Mr. A. S. Sieben for valuable technical assistance.

References

1. ALTERMATT, H. A., BLACKWOOD, A. C., and NEISH, A. C. The anaerobic dissimilation of D-xylose-1-C^{14}, D-xylose-2-C^{14}, and D-xylose-5-C^{14} by *Leuconostoc mesenteroides*. Can. J. Biochem. and Physiol. **33**, 622–626 (1955).
2. BATES, F. J. Polarimetry, saccharimetry and the sugars. U.S. Government Printing Office, Washington, D.C. 1942. p. 210.
3. BLACKWOOD, A. C. and BLAKLEY, E. R. Carbohydrate metabolism by *Leuconostoc mesenteroides*. Can. J. Microbiol. **2**, 741–746 (1956).
4. BLACKWOOD, A. C. and BLAKLEY, E. R. Metabolism of gluconate and 2-ketogluconate by *Leuconostoc mesenteroides* Bacteriol. Proc. 121 (1957).
5. BLAKLEY, E. R. and BLACKWOOD, A. C. The degradation of 2-keto-D-gluconate-C^{14}, D-gluconate-C^{14}, and D-fructose-C^{14} by *Leuconostoc mesenteroides*. Can. J. Microbiol. **3**, 741–744 (1957).
6. COHEN, S. S. Gluconokinase and the oxidative path of glucose-6-phosphate utilization. J. Biol. Chem. **189**, 617–628 (1951).
7. COLOWICK, S. P. and KALCKAR, H. M. The role of myokinase in transphosphorylations. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphate. J. Biol. Chem. **148**, 117–126 (1943).
8. CRANE, R. K. and SOLS, A. *In* Methods in Enzymology. Vol. I. *Edited by* S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York. 1955. p. 279.
9. DE LEY, J. The phosphorylation of some carbohydrates, connected with the direct oxidation, by *Aerobacter cloacae*. Enzymologia, **16**, 99–104 (1953).
10. DE LEY, J. 2-Keto-D-gluconate-6-phosphate, a new intermediate in the carbohydrate metabolism of *Aerobacter cloacae*. Enzymologia, **17**, 55–68 (1954).
11. DE LEY, J. Paper chromatographic detection and separation of 2-ketogluconate-phosphoric esters. Naturwiss. **42**, 96 (1955).
12. DE LEY, J. and VANDAMME, J. The metabolism of sodium 2-keto-D-gluconate by microorganisms. J. Gen. Microbiol. **12**, 162–171 (1955).
13. DE LEY, J. and VERHOFSTEDE, S. The place of 2-keto-D-gluconate-6-phosphate in carbohydrate metabolism. Enzymologia, **18**, 47–62 (1957).
14. DEMOSS, R. D. Routes of ethanol formation in bacteria. J. Cellular Comp. Physiol. **41**, Suppl. 1, 207–224 (1953).
15. DICKENS, F. Oxidation of phosphohexonate and pentose phosphoric acids by yeast enzymes. I. Oxidation of phosphohexonate. II. Oxidation of pentose phosphoric acids. Biochem. J. **32**, 1626–1644 (1938).
16. FEWSTER, J. A. Kinase activity of ultra-sonic extracts of *Acetobacter suboxydans*. Biochem. J. **66**, 9p (1957).
17. FISKE, C. H. and SUBBAROW, Y. The colorimetric determination of phosphorous. J. Biol. Chem. **66**, 375–400 (1925).
18. FRAMPTON, E. W. and WOOD, W. A. The mechanism of 2-keto-6-phosphogluconate degradation in *Pseudomonas fluorescens*. Bacteriol. Proc. 122 (1957).
19. FRAMPTON, E. W. Ph.D. Thesis, University of Illinois. 1958.
20. GHIRETTI, F. and GUZMAN BARRON, E. S. The pathway of glucose oxidation in *Corynebacterium creatinovorans*. Biochim. et Biophys. Acta, **15**, 445–460 (1954).
21. HANES, C. S. and ISHERWOOD, F. A. Separation of the phosphoric esters on the filter paper chromatogram. Nature, **164**, 1107–1112 (1949).
22. HOUGH, L., JONES, J. K. N., and WADMAN, W. H. Quantitative analysis of mixtures of sugars by the method of partition chromatography. V. Improved methods for the separation and detection of the sugars and their methylated derivatives on the paper chromatogram. J. Chem. Soc. 1702–1706 (1950).
23. KALCKAR, H. M. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem. **167**, 461–475 (1947).
24. KATZNELSON, H. Hexose phosphate metabolism by *Acetobacter melanogenum*. Can. J. Microbiol. **4**, 25–34 (1958).
25. LANNING, M. C. and COHEN, S. S. The detection and estimation of 2-ketohexonic acids. J. Biol. Chem. **189**, 109–114 (1951).
26. LIPMANN, F. Fermentation of phosphogluconic acid. Nature, **138**, 588–589 (1936).

27. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951).
28. LUGG, J. W. H. and OVERELL, B. T. Partition chromatography of organic acids on a paper sheet support. Nature, **160**, 87–88 (1947).
29. NARROD, S. A. and WOOD, W. A. Carbohydrate oxidation by *Pseudomonas fluorescens*. V. Evidence for gluconokinase and 2-ketogluconokinase. J. Biol. Chem. **220**, 45–55 (1956).
30. NELSON, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**, 375–380 (1944).
31. OHLE, H. and BEREND, G. 2-Keto-gluconsaüre. Ber. **60**, 1159–1167 (1927).
32. PALLERONI, N. J. and DOUDOROFF, M. Mannose isomerase of *Pseudomonas saccharophila*. J. Biol. Chem. **218**, 535–548 (1956).
33. PFEIFER, V. F., VOJNOVICH, C., HEGER, E. N., NELSON, G. E. N., and HAYNES, W. C. Production of calcium 2-ketogluconate by fermentation with species of *Pseudomonas*. Ind. Eng. Chem. **50**, 1008–1012 (1958).
34. SABLE, H. Z. and GUARINO, A. J. Phosphorylation of gluconate in yeast extracts. J. Biol. Chem. **196**, 395–402 (1952).
35. SIMPSON, F. J., WOLIN, M. J., and WOOD, W. A. Degradation of L-arabinose by *Aerobacter aerogenes*. I. A pathway involving phosphorylated intermediates. J. Biol. Chem. **230**, 457–472 (1958).
36. SOMOGYI, M. A new reagent for the determination of sugars. J. Biol. Chem. **160**, 61–68 (1945).
37. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques. Burgess Publishing Co., Minneapolis, Minn. 1957. p. 19.
38. WOOD, W. A. and SCHWERDT, R. F. Carbohydrate oxidation by *Pseudomonas fluorescens*. I. The mechanism of glucose and gluconate oxidation. J. Biol. Chem. **201**, 501–511 (1953).
39. YAMANAKA, K. Studies on the pyruvate and carbohydrate metabolisms by lactic acid bacteria. Part IX. Formation of ketopentoses by lactic acid bacteria. Bull. Agr. Chem. Soc. Japan, **22**, 299–308 (1958).

GROWTH STUDIES ON BACILLUS STEAROTHERMOPHILUS[1]

NORA E. NEILSON, MARY F. MACQUILLAN, AND J. J. R. CAMPBELL

Abstract

Growth curves for *B. stearothermophilus* were established for temperatures between 45° C and 70° C. Generation times as short as 10 minutes were obtained. At 65° C initial logarithmic growth was followed by autolysis of over 90% of the cells and subsequently by initiation of a second period of logarithmic growth. The effects of temperatures from 45° C to 64° C on the characteristics of the growth curves are almost identical with changes reported for *E. coli* between 22° C and 42° C.

Introduction

In spite of the generally accepted concept that thermophilic bacteria have rates of multiplication commensurate with the high temperatures at which they grow, data are not available to substantiate this suggestion. Hansen (3) concluded that the fermenting capacity of a thermophile at 55° C was 30 times that of a lactic streptococcus at around 20° C. However, the generation time established for the thermophile at 55° C was 16 minutes which probably represents only a fivefold increase over that of the streptococcus at 20° C. The growth rate of the thermophile, therefore, was not nearly as rapid, in relation to the mesophile, as one would have predicted from their relative metabolic activities. Imsenecki and Solnzeva (4), Militzer *et al.* (6), Allen (1), Hancock (2), and others have recorded growth data for a variety of thermophiles. However, the generation times calculated from their data indicate that the organisms under study had generation times greatly in excess of the 16 minutes recorded by Hansen. It is now known that many earlier workers underestimated the importance of adequate aeration of the cultures, the need for neutralizing acid formed, and the rapid rate of evaporation of the medium at high temperatures. Moreover, Neilson, MacQuillan, and Campbell (8) found that when routine plating procedures were used for the determination of numbers of thermophiles, only a fraction of the population formed colonies. In the present work, precautions were taken to control these factors and growth curves at various temperatures were established for a strain of *Bacillus stearothermophilus*.

Methods

The general growth and plating conditions were those previously determined by Neilson, MacQuillan, and Campbell (8). The culture used was strain B44 of *Bacillus stearothermophilus* described by Neilson and Christie (7). The growth medium contained tryptic digest of casein, 10% by volume of a 9% solution; Bacto yeast extract, 0.5%; K_2HPO_4, 0.25%; glucose, 0.1%;

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Contribution from the Dairying Laboratory, The University of British Columbia, Vancouver 8, B.C., Canada.

TABLE I

Adequacy of medium for maximum growth of *B. stearothermophilus*

	Hours											
	0	½	1	1½	2	2½	3	3½	4	4½	5	6
	Viable cells per ml medium, $\times 10^6$											
Half strength broth	.015	.014	.053	.18	1.45	11	75	152	167	300	430	770
Single strength broth	.013	.015	.042	.15	.93	6.5	64	320	500	480	600	1100
Double strength broth	.012	.013	.027	.21	.51	4.2	17	155	580	760	1030	1150

pH 7.2. The plating medium was Bacto plate count agar and the plating diluent was 0.8% KCl in *M*/200, pH 7.2, phosphate buffer at 37° C. Plate counts were carried out in duplicate and incubation was at 55° C for 16 hours. A 16-hour culture grown at 45° C served as the inoculum and was added to give an initial count of about 20,000 viable cells per ml.

Results

In order to establish that the concentrations of nutrients in the growth medium were not limiting, viable cell counts were followed on cultures grown in half, single, and double strength broths (Table I).

The growth rates in the three media were similar up to a cell count of about 1×10^8 per ml. At this point the growth rate in the half strength medium changed to a slower but constant rate until the cell count reached almost 1×10^9 cells per ml. In the other two media, the initial rate continued to a count of 5×10^8 cells per ml at which time the rate decreased gradually until a maximum viable count of about 1×10^9 cells per ml was achieved. It would appear, therefore, that the use of the single strength broth in future experiments would ensure that nutrients were not limiting in cell multiplication. It was also established that aeration was not limiting, that no evaporation occurred during the experiment, and that the pH of the medium did not go below 7.0.

The representative data presented in Fig. 1 illustrate the growth curves obtained at temperatures from 45° C to 70° C. At 65° C to 70° C growth was rapid for about 3 hours and the population reached an average of 2×10^8 cells per ml. At this time rapid autolysis of the cells occurred as indicated by a marked clearing of the medium and by a rapid drop in viable cell count. As incubation of the culture was continued, however, the cell numbers increased once more and eventually reached approximately 1×10^9 cells per ml,

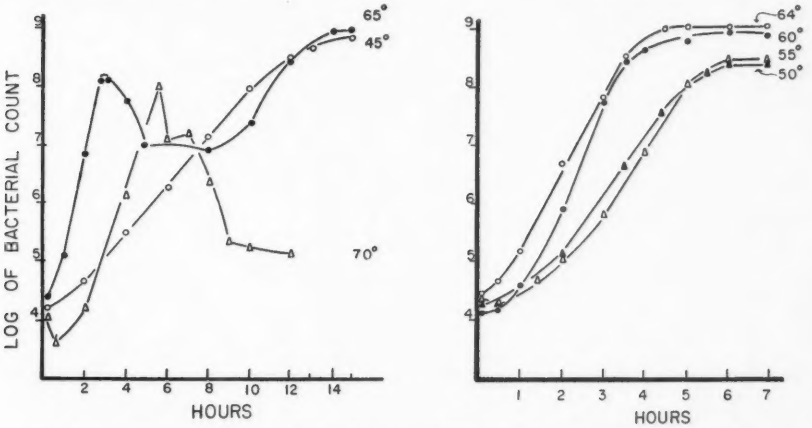
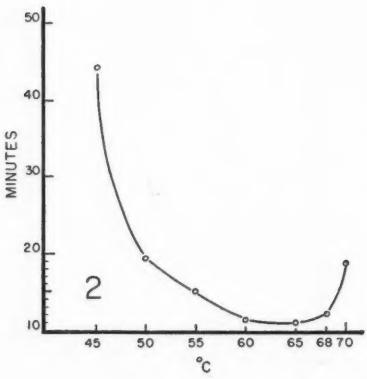


FIG. 1. Growth curves of *B. stearothermophilus* at various temperatures. Counts were made at 15-minute intervals for the first 2 hours and at ½-hour intervals thereafter.

the maximum number achieved with cultures at lower temperatures. Contrary to our expectations, but in agreement with the observations of Jennison (5), repeated transfer of the inoculum at a particular temperature did not change the growth curves obtained in Fig. 1. One might assume that a culture which had survived the period of autolysis and had continued to multiply rapidly would have a higher optimum temperature than the original culture and would not go through this autolytic cycle on subculturing at 65° C. However, it was found that repeated transfer of the inocula at temperatures of 45° C to 65° C did not influence the shape of the growth curves, the optimum temperature of growth, or the autolytic cycle. The generation times during the exponential growth phase at each of the temperatures were calculated and are presented as average values in Fig. 2. As may be seen, between 60° C and 68° C this microorganism has an average generation time of 12 minutes. Values as low as 10 minutes have been obtained on a number of occasions. In contrast to findings of previous workers, the culture maintained a near maximum population of 1×10^9 cells per ml at 60° C for 21 hours before decreasing to 2×10^8 cells at 48 hours (Fig. 3). Similar data were obtained at 55° C. Even at 65° C a high viable count was maintained for at least 36 hours. The growth curves of this thermophile are basically very similar to those described by Jennison (5) for *Escherichia coli*. The curves for the respective organisms show the same ratio of approximately 5:1 between generation time in minutes and the logarithmic growth period in hours. The Q_{10} varies between 2.7 and 1.0 and the lag phase is particularly brief and sharply defined towards the maximum temperatures of growth. Jennison's work with *E. coli* was carried out over the range 22° C–42° C while our comparisons with *B. stearothermophilus* were over the range 45° C–64° C.



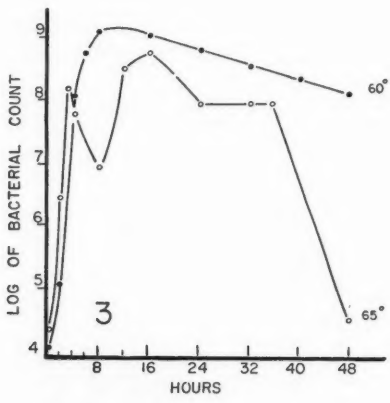


FIG. 2. Generation times of *B. stearothermophilus*.
FIG. 3. Growth curves of *B. stearothermophilus* on prolonged incubation.

References

1. ALLEN, M. B. The thermophilic aerobic spore forming bacteria. Bacteriol. Rev. **17**, 125–173 (1953).
2. HANCOCK, R. Protein synthesis in a species of thermophilic Bacillus. J. Gen. Microbiol. **17**, 480–490 (1957).
3. HANSEN, P. A. The growth of thermophilic bacteria. Arch. Mikrobiol. **4**, 25–35 (1933).
4. IMSENECKI, A. and SOLNZEVA, L. The growth of aerobic thermophilic bacteria. J. Bacteriol. **49**, 539–546 (1945).
5. JENNISON, M. W. Some quantitative relationships in bacterial population cycles. J. Bacteriol. **30**, 603–623 (1935).
6. MILITZER, W., SONDEREGGER, T. B., and TUTTLE, L. C. Thermal enzymes. Arch. Biochem. **24**, 75–82 (1949).
7. NEILSON, N. E. and CHRISTIE, H. W. *Bacillus stearothermophilus* in herring stickwater. Can. J. Microbiol. **3**, 937–938 (1957).
8. NEILSON, N. E., MACQUILLAN, M. F., and CAMPBELL, J. J. R. The enumeration of thermophilic bacteria by the plate count method. Can. J. Microbiol. **3**, 939–943 (1957).

FLUORESCENCE OF AZOTOBACTER

I. A COMPARISON OF THE FLUORESCENT PIGMENTS WITH RIBOFLAVIN[1]

D. B. JOHNSTONE, M. PFEFFER,[2] AND G. C. BLANCHARD[2]

Abstract

The fluorescent pigments elaborated in cultures of certain species of *Azotobacter* were compared with riboflavin produced by the same cultures. No correlation between the amounts of fluorescence and riboflavin was observed. Iron appears to inhibit the synthesis of the fluorescent pigments but stimulates riboflavin production. The fluorescent pigments obtained from *Azotobacter* were water-soluble, thermostable, insoluble in water-free organic solvents, and each exhibited a single absorption peak in the region of 370–380 mμ in slightly acid solution shifting to 400–420 in alkaline solution. Partial purification has been achieved by continuous paper electrophoresis with an acid electrolyte. These studies appear to indicate that the marked fluorescence observed in certain *Azotobacter* cultures cannot be attributed to riboflavin.

Introduction

The synthesis of water-soluble substances that fluoresce upon excitation by ultraviolet light of 3600 Å is confined, in the genus *Azotobacter*, to three species, *A. agilis*, *A. vinelandii*, and *A. macrocytogenes* (4). These fluorescent substances are thermostable and insoluble in water-free organic solvents. Interest in the ability to distinguish species by the wave length or color of the fluorescence prompted investigation of the chemical nature of the fluorescent material (6).

Azotobacter species have been the object of extensive investigations into riboflavin synthesis and have been considered by some to be a rich source of this vitamin. Lee and Burris (7), during studies of large-scale production methods, obtained higher yields of riboflavin from *A. vinelandii* than from brewer's yeast; and Pridham (9), in a survey of riboflavin-synthesizing microorganisms, concluded that the genus *Azotobacter* was one of three genera offering promise as a rich source of riboflavin. It is of interest that Starkey (10) recorded the fluorescent pigmentation of *Azotobacter* in a table of riboflavin values.

We have used pH–fluorescence curves (5) to distinguish the fluorescent pigments of *A. vinelandii* and *A. agilis*. The pH–fluorescence curves of riboflavin and *Azotobacter* pigments show a marked similarity. Thus, the possibility existed that the fluorescence observed in *Azotobacter* cultures might be due largely to an accumulation of this vitamin in the medium. This paper deals with an investigation of this possibility as well as with attempts to characterize the material responsible for the fluorescence.

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Materials and Methods

Strains of *Azotobacter* specifically referred to in the paper are *A. agilis* strain 132, ATCC 12838; *A. vinelandii* strain 3A, ATCC 12837; and *A. chroococcum* ATCC 9544. The first two strains were isolated in this laboratory from water and soil respectively.

Several strains of various species of *Azotobacter* were grown in a Burk's liquid, nitrogen-free, mineral salt medium (12) both with and without the addition of iron. After about 7 days' incubation at 30° C in 250-ml Erlenmeyer flasks on a rotary shaking machine, the cultures were centrifuged. The supernatant medium was analyzed microbiologically for riboflavin and the relative fluorescence was measured in a fluorimeter. *Lactobacillus casei* ATCC 7469 was employed in the assay of riboflavin. This organism was cultured in Bacto micro inoculum broth. The microbiological assay for riboflavin was carried out with Bacto riboflavin assay medium according to the procedure outlined in the Difco manual (1). Relative fluorescence of various culture filtrates was obtained by the use of a Photovolt fluorimeter according to a procedure previously reported (4).

Absorption curves were obtained with the use of a Beckman DU spectrophotometer employing aqueous solutions. Electrophoretic data were obtained with a Spinco model CP continuous paper electrophoresis apparatus. The electrolyte was 5% acetic acid, the feed rate 7 ml per hour, the current 60 milliamps, and the voltage approximately 500 volts.

Results

Figure 1 illustrates the results observed with three representative species. Duplicate sets designated by Fe are presented for each species showing the effect of adding 1 p.p.m. of iron in the form of ferric chloride to a relatively iron-free medium. The most fluorescent culture shown here, *A. agilis*, with no iron added, contained the least amount of riboflavin. The addition of iron increased the riboflavin content. Though iron is considered essential for riboflavin synthesis, at some concentrations it is known to strongly inhibit riboflavin production by other genera (3). With the addition of iron, fluorescence decreased. This might have been expected, for the synthesis of fluorescent pigments by pseudomonads is also inhibited by iron (11).

A sharp contrast was also noted in the cultures of *A. vinelandii*, where the iron markedly stimulated riboflavin and decreased the fluorescence. *A. chroococcum* was included to provide a non-fluorescent species for purposes of comparison and it elaborated riboflavin in amounts comparable with the other two species. By adding iron to fluorescent culture filtrates, it was observed that iron does not quench the fluorescence but rather inhibits the synthesis of the compound which fluoresces.

In all instances, the riboflavin was present in insufficient concentration to be measured as fluorescence. That is, if one were to add 1 μg of riboflavin per ml of medium, the greatest amount shown here, and if the fluorimeter

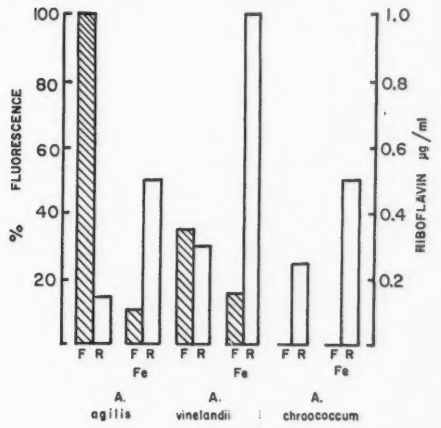

FIG. 1. Comparison of the per cent of relative fluorescent intensity with the amount of riboflavin in cell-free culture filtrates of three species of *Azotobacter*. Shaded vertical bars designated by F indicate fluorescence. Clear vertical bars designated by R indicate riboflavin content. The pairs of bars designated by Fe are those cultures to which iron was added.

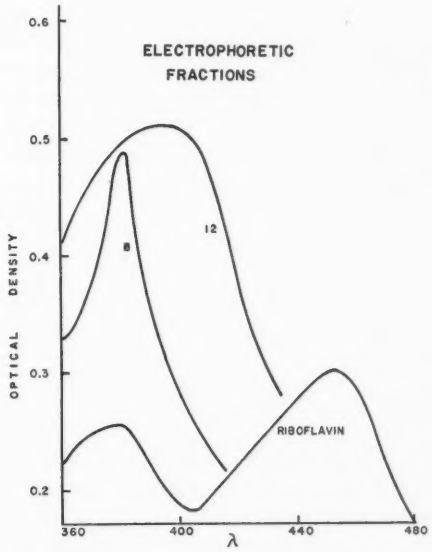

FIG. 2. Absorption curves of two electrophoretic fractions from a culture filtrate of *A. vinelandii*. Fractions 8 and 12 are from tabs 8 and 12 on a continuous paper electrophoresis curtain. The absorption curve of riboflavin is included for reference.

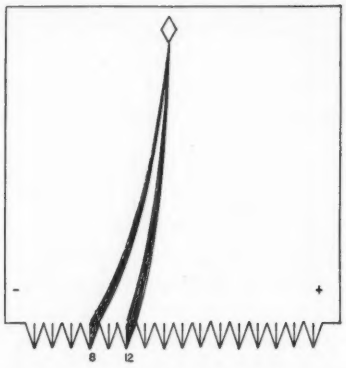

FIG. 3. Bands of fluorescence on a continuous paper electrophoresis curtain. Under an ultraviolet lamp, 8 fluoresces white and 12 fluoresces yellow.

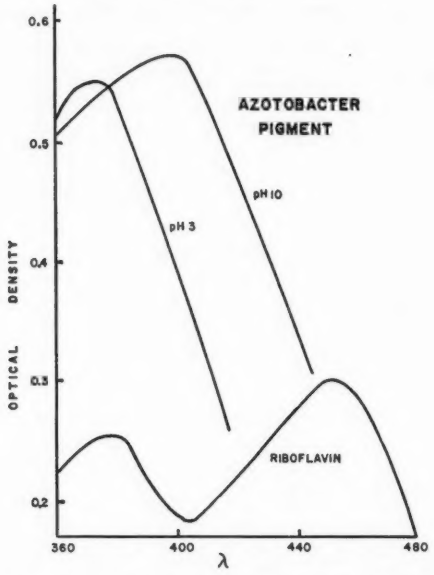

FIG. 4. Absorption curves of *Azotobacter* fluorescence at pH 3 and pH 10. Riboflavin is included for reference.

were set to read 100 with the highly fluorescent *A. agilis* culture filtrate, the riboflavin would not show any fluorescence. It is not until one sets the fluorimeter at a much lower range that the fluorescence of the riboflavin becomes measurable. Thus, even though this *A. chroococcum* filtrate has from 0.2 to 0.5 μg of riboflavin per ml, it is still insufficient to be measured here as fluorescence. Therefore, it does not appear that the marked fluorescence observed in certain *Azotobacter* cultures can be attributed to riboflavin.

The question then remained as to whether one was dealing here with another flavin. The moiety of riboflavin responsible for its characteristic absorption spectrum is the iso-alloxazine nucleus. More complex riboflavin derivatives may have modified absorption spectra but usually display the characteristic double peak in the region of 350 to 470 mμ. Employing crude culture filtrates of *A. vinelandii*, Wilson (13) observed a single peak in this region. This has been confirmed in our laboratory with sufficiently good peaks to rule out the possibility of a double peak over a wide range, as is characteristic of riboflavin. Moreover, fluorescent fractions of *A. vinelandii* obtained by continuous paper electrophoresis again confirmed single peaks in this region as illustrated in Fig. 2. Riboflavin is included for reference. The fractions 8 and 12 from tabs 8 and 12 on a continuous paper electrophoresis curtain were neutralized before the absorption spectra were obtained. Figure 3 shows diagrammatically the two fluorescent bands as observed under ultraviolet light. That terminating at tab 8 appeared to fluoresce white and that at 12 fluoresced yellow.

Flavins are characterized by little or no shift in absorption peaks due to pH change, whereas the fluorescent pigment from *Azotobacter* does show such a shift. Figure 4 indicates the extent of this shift from pH 3 to a higher wave length at pH 10. Riboflavin is again included for reference.

Discussion

Various methods have been employed for purifying flavins, notably precipitation by silver, lead, iron, mercury, or similar heavy metals as well as by the use of chromatographic techniques. Attempts to recover the fluorescent pigments of *Azotobacter* by such methods have failed. Adsorption of the material has been achieved on a variety of adsorbents such as charcoal, alumina, Fuller's earth, etc., but attempts to elute with such eluants as employed for riboflavin have proved inadequate. It seems very likely that if this material were a flavin its identity would be apparent in such examinations.

From the viewpoint of absorption spectra, the pteridine derivatives are suggested, since they seem to show similar absorption peaks which shift with pH changes in the same manner as was observed with our material. It is of interest to note that extracellular fluorescent substances elaborated by certain aspergilli, which were once thought to be riboflavin, have characteristics which indicate they are probably pteridine derivatives (14). Moreover, Nathan, Hutner, and Levin (8) have indicated a connection between riboflavin and pteridine-containing vitamins and proposed a scheme to illustrate interrelationships of folic acid, riboflavin, and crithidia factor. The fluorescent substance pyoverdine, elaborated by pseudomonads and studied recently by Elliott (2), appears to resemble our data of the fluorescent material from *Azotobacter*. Both substances showed absorption peak shifts with changes in pH whereas riboflavin did not.

Although no role can yet be attributed to this fluorescent pigment synthesis, its existence offers an area for study. Since its production can be controlled by alteration of the medium, and its presence is not essential to the bacterial cells capable of its elaboration, one may study experimentally both its presence and absence. One use to which the fluorescence may be put, in addition to identifying species, is that of a genetic marker. Such studies are currently in progress.

References

1. DIFCO MANUAL, 9th ed. Difco Laboratories, Inc., Detroit. 1953.
2. ELLIOTT, R. P. Some properties of pyoverdine, the water-soluble fluorescent pigment of the pseudomonads. Appl. Microbiol. **6**, 241–246 (1958).
3. ENARI, T. M. Studies on the uptake of cobalt and iron and their effect on the production of riboflavin by *Candida guilliermondii*. Ann. Acad. Sci. Fennicae. Ser. A, II, 1–42 (1958).
4. JOHNSTONE, D. B. The use of a fluorimeter in the characterization of fluorescing substances elaborated by *Azotobacter*. Appl. Microbiol. **5**, 103–106 (1957).
5. JOHNSTONE, D. B. and FISHBEIN, J. R. Identification of *Azotobacter* species by fluorescence and cell analysis. J. Gen. Microbiol. **14**, 330–335 (1956).
6. JOHNSTONE, D. B., PFEFFER, M., and BLANCHARD, G. C. Fluorescence of *Azotobacter*, its chemical nature and bacteriological significance. Intern. Congr. Microbiol. Abstracts, 7th Congr. 386–387 (1958).
7. LEE, S. R. and BURRIS, R. H. Large scale production of *Azotobacter*. Ind. and Eng. Chem. **35**, 354–357 (1943).
8. NATHAN, H. A., HUTNER, S. H., and LEVIN, H. L. Independent requirements for "Crithidia Factor" and folic acid in a trypanosomid flagellate. Nature, **178**, 741–742 (1956).
9. PRIDHAM, T. G. Microbial synthesis of riboflavin. Econ. Botany, **6**, 185–205 (1952).
10. STARKEY, R. L. Changes in the content of certain B-vitamins in organic materials decomposing under aerobic and anaerobic conditions. Soil Sci. **57**, 247–270 (1944).
11. TOTTER, J. R. and MOSELEY, F. T. Influence of the concentration of iron on the production of fluorescin by *Pseudomonas aeruginosa*. J. Bacteriol. **65**, 45–47 (1953).
12. WILSON, P. W. and KNIGHT, S. G. Experiments in bacterial physiology. Burgess Publishing Co., Minneapolis, Minn. 1952.
13. WILSON, T. G. G. Physical chemical researches on bacteria. Thesis, London University. 1952.
14. WOLF, F. T. The fluorescent pigment of *Aspergillus repens*. Physiol. Plantarum, **10**, 825–831 (1957).

STUDIES WITH KANAMYCIN AGAINST MYCOBACTERIUM TUBERCULOSIS[1]

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Abstract

The original Japanese observations on kanamycin are confirmed as to activity in vitro and in mice. They are extended to include: (*a*) cross-resistance studies, (*b*) experiments with kanamycin A and B singly and together, (*c*) combined therapy experiments with other tuberculostatic agents.

Samples of kanamycin A and A plus B were of the same order of activity as streptomycin in vitro and in mice, and were effective against streptomycin-resistant organisms. Kanamycin B did not increase mouse survival when tested at a level that with kanamycin A gave good prolongation of life. When mice were treated with combinations of kanamycin plus streptomycin, or dihydrostreptomycin, or neomycin, the survival increases obtained were greater than were to be expected from the mere sum of the effects of the individual antibiotics.

Introduction

Kanamycin, a new antibiotic, was isolated by Umezawa and co-workers (4) from a streptomyces named by them *Streptomyces kanamyceticus*. Its activity against tubercle bacilli in vitro and in mice was established by Yanagisawa and Sato (5) and its effect in guinea pigs by Yanagisawa *et al.* (6). The present report confirms and extends the findings of the aforementioned authors relating to in vitro and mouse experiments. In addition, some combined therapy experiments have been performed, and comparisons made between samples of kanamycin A and B, and mixtures of the two components.

Methods

The medium of Dubos containing albumin was used throughout. In vitro assays were performed by making serial twofold dilutions of kanamycin in 0.5 ml of water, adding 2 ml of medium containing H37Rv at a 10^{-2} dilution, and incubating at 37° C for 7 days. To study the development of resistance, cultures were carried through 10 successive transfers at weekly intervals in the presence of kanamycin. Streptomycin was run concurrently. Each transfer was made from the highest concentration of antibiotic permitting 2 plus growth or better. The inoculum in this case was a 1:50 dilution of a 7-day-old culture of H37Rv. For the in vivo experiments, mice of the CF_1 strain weighing 16–17 g were infected intravenously with 0.25 ml of a culture of H37Rv or its resistant variants grown in Erlenmeyer flasks for 5 days. The inoculum was standardized at 65–70 in the Klett-Summerson colorimeter and further diluted 1:4 with Dubos-albumin medium. Inoculum prepared in this manner is fatal to control mice in 18 to 20 days. All animals received ground Purina diet, and were weighed at weekly intervals. Kanamycin,

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streptomycin, and neomycin were given by the subcutaneous route; isonicotinic acid hydrazide, pyrazinamide, and *p*-aminosalicylic acid were administered in the feed. Treatment was begun the day of infection and continued for only 7 days since we have shown that most agents effective in murine tuberculosis give good survival time increases with this limited period of treatment (1).

Results

In Vitro

Samples of kanamycin A or mixtures of A plus B gave complete inhibition of growth at levels of .078 to .156 μg/ml against the susceptible strain of H37Rv as well as its streptomycin-resistant and isonicotinic acid resistant variants. This was in the same range as streptomycin and better than neomycin. Kanamycin B was less active. On the other hand, a neomycin-resistant strain of H37Rv, though susceptible to streptomycin, was not inhibited by kanamycin (Table 1). Two chromogenic acid fasts of human origin were inhibited by an amount of kanamycin 20 times less than the level of streptomycin required to prevent growth. The bactericidal end point, as with streptomycin, was found to be two to four times the bacteriostatic end point. No cross resistance was found between streptomycin and kanamycin, nor was there any synergism between kanamycin and streptomycin, *p*-aminosalicylic acid or isonicotinic acid hydrazide. Autoclaving for 15 minutes at 15 lb did not alter the bacteriostatic activity of either agent.

TABLE I

In vitro inhibition of susceptible and resistant strains of H37Rv by kanamycin

Agent	No. of samples	μg/ml required for complete inhibition of *				
		H37Rv	H37 SMR	H37 INHR	H37 KMR	H37 NeoR
Kanamycin A + B	10	.078–.156	.078	.156	>40	>500
Kanamycin A	7	.078–.156	.078	.156	>40	>500
Kanamycin B	3	.31–1.25	.62–1.25	.62–1.25	>40	>500
Streptomycin	4	.078	20	.156	.078	.156
Neomycin	1	.27	.62	1.25	>40	>1000

*H37 SMR = H37Rv culture resistant to streptomycin;
H37 INHR = H37Rv culture resistant to isonicotinic acid hydrazide;
H37 KMR = H37Rv culture resistant to kanamycin;
H37 NeoR = H37Rv culture resistant to neomycin.

The *rate* of development of resistance to kanamycin was found to be a little slower than that to streptomycin. This was true whether the material contained A and B or only kanamycin A. At the fourth passage, resistance had begun to develop to streptomycin, but not to kanamycin. However, after 10 successive transfers there was an eightfold increase for both streptomycin and the kanamycins in the amount required for complete inhibition of growth (Table II).

TABLE II

Development of resistance to kanamycin and streptomycin*

	Tube No.									
	1	2	3	4	5	6	7	8	9	
	Index of resistance									Passage
Kanamycin	0	0	0	0	0	0	±	2	4	II
	0	0	0	0	0	0	±	2	4	IV
	0	0	0	0	±	3	4	4	4	VII
	0	0	0	±	3	4	4	4	4	VIII
Streptomycin	0	0	0	0	0	0	0	1	4	II
	0	0	0	0	0	1	2	3	4	IV
	0	0	0	0	0	2	4	4	4	VI
	0	0	0	0	±	3	4	4	4	VIII
μg/ml	5	25	1.25	.62	.31	.15	.078	.039	.019	

*0 = no growth; 4 = full growth.

In Vivo

All of the kanamycin A and A + B samples tested were as effective and usually more effective in prolonging the survival of infected mice than was streptomycin at equivalent doses. Neomycin was less active than either agent. However, three kanamycin B samples tested under the same conditions showed no activity (Table III). (The therapeutic effectiveness of higher levels was not investigated because of the greater toxicity for normal animals of B compared with A samples of kanamycin.)

TABLE III

In vivo activity of kanamycins against H37Rv susceptible strain, 7 days' treatment

Treatment	No. of samples	Mg/day s.c.	Days' increased survival*
Kanamycin A + B	3	2.0	>20
Streptomycin	3	2.0	14–19
Neomycin	1	2.0	11.0
Kanamycin A	7	1.0	7.5 to >20.0
Kanamycin B	3	1.0	0
Kanamycin A + B	3	1.0	9.5 to >20
Streptomycin	3	1.0	5–9
Neomycin	1	1.0	7.0

NOTE: Control survival time: 18.8–19.6 days.
*Experiments terminated 40th day after infection.

Two samples of kanamycin (A + B) were tested in mice infected with a streptomycin-resistant strain of H37Rv. While streptomycin was without significant effect, the kanamycin treatment gave prolongations of survival time of the order obtained in mice infected with a susceptible strain of H37Rv (Table IV). Kanamycin was also found to prolong survival in mice infected with a strain of H37Rv resistant to isonicotinic acid hydrazide (Table IV).

TABLE IV

In vivo activity of kanamycin against H37Rv resistant strains

Treatment*	Infection	Days' increased survival	Control survival time
Kanamycin	H37Rv streptomycin resistant	>16.0	18.7
Streptomycin	H37Rv streptomycin resistant	2.0	18.7
Kanamycin	H37Rv isonicotinic acid resistant	>10	30.0

*1 mg/day for 7 days.

These therapeutic effects bear out the in vitro findings of the lack of cross resistance of kanamycin with streptomycin and isonicotinic acid hydrazide.

In order to investigate the effect of combined therapy, tuberculous mice were treated with combinations of kanamycin plus streptomycin or dihydrostreptomycin or neomycin; kanamycin plus isonicotinic acid hydrazide; kanamycin plus pyrazinamide; and kanamycin plus *p*-aminosalicylic acid. Barely effective quantities of streptomycin, dihydrostreptomycin, or neomycin were much more effective in prolonging survival when they were combined with kanamycin. An effect was obtained that was two to four times that of the sum of the increased survivals resulting from treatment with the individual agents. In contrast, the results obtained with isonicotinic acid hydrazide, *p*-aminosalicylic acid, and pyrazinamide plus kanamycin were not significantly different from the sum of the effects of the individual components (Table V).

TABLE V

Combined therapy experiments with kanamycin

Treatment	Dose/day, mg	Days' increased survival
Kanamycin A43	0.25	5.4
Neomycin	0.5	4.2
Dihydrostreptomycin	0.5	4.6
Kanamycin + neomycin	0.25 + 0.5	>19.0
Kanamycin + dihydrostreptomycin	0.25 + 0.5	>18.0
Kanamycin A13	0.5	5.2
	0.25	0
Streptomycin	1.0	5.1
	0.5	4.2
Kanamycin A13 + streptomycin	0.5 + 1.0	22.4
	0.25 + 0.5	14.3
Isonicotinic acid hydrazide	0.1	8.3
	0.05	0
Kanamycin A13 + isonicotinic acid hydrazide	0.5 + 0.1	14.3
	0.25 + 0.05	4.4
Kanamycin A42	0.25	8.2
Pyrazinamide	10	11.8
Kanamycin A42 + pyrazinamide	0.25 + 10	7.8
p-Aminosalicylic acid	40	6.8
Kanamycin A42 + *p*-aminosalicylic acid	0.25 + 40	15.0

Comment

During the course of these experiments, in vitro results were reported by others in which higher levels of kanamycin were required for complete inhibition of growth than those found by us (2, 3). One microgram per milliliter was the end point in the original communication of Yanagisawa and Sato (5). The types of media used and the inoculum size may account for these differences, or the discrepancy may lie in the different lots of antibiotic employed by various workers.

Yanagisawa and co-workers concluded that kanamycin had the same order of activity as streptomycin on the basis of numbers of organisms recovered from infected mouse tissues (5). Judging effectiveness by survival time, it would appear that it has greater therapeutic activity. Most samples tested by us at a dose of 1.0 mg for seven doses, a shorter period of treatment than that employed by the Japanese workers, were more effective than streptomycin. The complete ineffectiveness of kanamycin B samples was surprising because, though in vitro activity was two to four times less than A samples, most of the latter gave some increased survival when doses of 0.5 and 0.25 mg were used.

Conclusions

Under the conditions described above, kanamycin A and A + B

(*a*) are equivalent to streptomycin in bacteriostatic and bactericidal power in vitro;

(*b*) are active against streptomycin-resistant and isonicotinic acid resistant strains;

(*c*) do not rapidly induce the development of resistance in the tubercle bacillus;

(*d*) are as good as and frequently better than streptomycin in prolonging survival of infected mice.

Three samples of kanamycin B were less active in vitro than A or A + B samples and were without therapeutic effect in mice at the level tested.

A greater than additive effect was obtained when infected mice were treated with a combination of kanamycin plus streptomycin, or dihydrostreptomycin, or neomycin.

References

1. FitzPatrick, F. K. Short-term therapy in experimental tuberculosis. Am. Rev. Tuberc. Pulmonary Diseases, **77**, 867–868 (1958).
2. Gourevitch, A., Hunt, G. A., and Lein, J. Antibacterial activity of kanamycin. Antibiotics & Chemotherapy, **8**, 149–159 (1958).
3. Patnode, R. A. and Hudgins, P. C. Effect of kanamycin on mycobacterium tuberculosis *in vitro*. Am. Rev. Tuberc. Pulmonary Diseases, **78**, 138–139 (1958).
4. Umezawa, H. *et al.* On a new antibiotic, kanamycin. J. Antibiotics (Japan), Ser. A, **10**, 181–188 (1957).
5. Yanagisawa, K. and Sato, N. Studies on kanamycin, a new antibiotic against tubercle bacilli. I. Effect on virulent tubercle bacilli *in vitro* and in mice. J. Antibiotics (Japan), Ser. A, **10**, 233–235 (1957).
6. Yanagisawa, K., Kanai, K., and Tachibana, T. Studies on kanamycin, a new antibiotic against tubercle bacilli. II. A preliminary guinea pig experiment. J. Antibiotics (Japan), Ser. A, **10**, 236–241 (1957).

NOTES

A MODIFIED TECHNIQUE FOR ISOLATION OF BACTERIOPHAGE FROM CONTAMINATED MATERIALS[1]

F. D. Cook and C. Quadling

This communication describes a modification of well-known techniques for the isolation of bacteriophages. Methods usually depend on the culturing of given host bacteria with source material, e.g. soil (3), sewage (2), or plant residues (5), in which the presence of phage is suspected. One expects phage particles present to multiply at the expense of the added host cells so that single plaque isolates may readily be obtained. Such mass-selection methods are appropriate when the aim is to obtain a virulent phage for diagnostic (5) purposes. There exists the possibility that other phages might not be detected if they were, for example, of low virulence, slow to absorb to the host, small burst size, long latent period, or lysogenizing rather than lytic. We tried to detect temperate phage in samples of arable soils by a modification of the mass-selection technique involving (*a*) limited opportunities for multiplication of infected bacteria or of already-present phages in the enrichment culture and (*b*) antibiotic-resistant host bacteria together with appropriate antibiotics to reduce microbial contamination which might otherwise obscure rare plaques in soft agar layer plates (1).

One strain each of *Rhizobium trifolii* (RT2), *R. meliloti* (RM6), *R. leguminosarum* (RL5), *R. phaseoli* (RP2), and *Xanthomonas phaseoli* (XP8) was used. Medium Y (6) was employed for the growth of *Rhizobium* and antibiotic medium 3 (Difco) for *Xanthomonas*. Mutants resistant to 2000 μg/ml of streptomycin (calcium chloride complex, Merk) were obtained by single-step selection on appropriate solid media containing streptomycin. Soil samples were obtained from the legume-crop nursery plots, Central Experimental Farm, Ottawa, and stored in screw-cap jars. In experiments, about 50 g of field soil were enriched with a total of approximately 5×10^9 bacteria, of one of the mutant strains mentioned above, suspended in 5 ml of antibiotic-containing liquid medium (500 μg/ml streptomycin). This mixture of soil with host bacteria, hereafter referred to as the enriched soil, was incubated for 16 hours to allow absorption and limited multiplication of soil-borne phages. Since we have never found phage titers greater than 10^5/g, and often much less, after enrichment, we assume that the number of cycles of phage infection, liberation, and reinfection of phage-sensitive cells is probably small.

After 16 hours at room temperature (approximately 25° C) 2 g of the enriched soil were suspended in 10 ml liquid medium for 2 hours. Particles of soil and vegetable debris were then sedimented by low-speed centrifugation and the supernatant tested for the presence of phage by plating 0.1-ml and 0.5-ml samples in soft agar layer (1) together with a culture of the appropriate

[1]Contribution No. 479, from the Bacteriology Division, Canada Department of Agriculture.

indicator bacterium. Streptomycin (500 μg/ml) was added to the basal layer of nutrient agar. Although antibiotic-resistant microorganisms, other than the added host bacteria, were always present they did not interfere with plaque formation. In some experiments tetracycline or chloramphenicol, in combination with appropriate resistant indicator bacteria, were used to control contamination with satisfactory results.

Twelve samples of local arable soils were tested: at least four lytic phages, active on *Rhizobium trifolii*, *R. meliloti*, *R. leguminosarum*, and *R. phaseoli* respectively, were isolated. The phage (PRP2) for *R. phaseoli* produced irregular plaques similar to those termed "star mutants" (4, 7). Three phages for *Xanthomonas phaseoli* were isolated. These were weakly lytic in that plaques were obtained in soft agar layer but little or no lysis was evident when the phage was cross-streaked against the host on an agar surface.

The recovery of freshly added phage from soil is reasonably efficient as was shown by control experiments in which about 2.4×10^3 infective particles contained in 0.1 ml of an arbitrarily selected phage stock were mixed with 3 g of air-dried soil, previously shown to be free from this phage. After 3 hours the phage was eluted from a 1-g sample of the mixture, as described above. When assayed by the soft agar layer method numbers of plaques representing between 80% and 90% of the added phage were found. Anderson (2) found that he could detect phage when a minimum was present of about 10^2 phage particles/ml in sewage or about 10^3 particles/ml in faeces. In one experiment with the soil samples mentioned above, no phage for *X. phaseoli* was detected in the absence of enrichment but phage was found in 3/12 samples after enrichment.

The technique described was designed to permit the isolation of temperate phages; in this we have been unsuccessful. However, this method may be of value for the isolation of phages for various purposes. Conceivably, it could be used to investigate the distribution of phages in soil or to determine indirectly the distribution of various bacteria, assuming that the distribution of phages approximates that of their specific hosts.

1. ADAMS, M. H. Methods of study of bacterial viruses. *In* Methods in medical research. Vol. 2. *Edited by* J. H. Comroe. Year Bk. Pubs. Inc., Chicago. 1950.
2. ANDERSON, E. S. *In* Microbial Ecology. *Edited by* R. E. O. Williams and C. C. Spicer. Cambridge University Press, London. 1957.
3. GROSS, J. E. and HINGORANI, M. K. A method for isolating *Pseudomonas mors-prunorum* phages from the soil. Nature, **181**, 60–61 (1958).
4. HERSHEY, A. D. Spontaneous mutations in bacterial viruses. Cold Spring Harbor Symposia on Quant. Biol. **11**, 67–76 (1946).
5. KATZNELSON, H. and SUTTON, M. D. A rapid plaque count method for the detection of bacteria as applied to the demonstration of internally borne bacterial infections of seed. J. Bacteriol. **61**, 680–701 (1951).
6. LOCHHEAD, A. G. and CHASE, F. E. Qualitative studies of soil microorganisms: V. Nutritional requirements of the predominant bacterial flora. Soil Sci. **55**, 185–195 (1943).
7. MCFALL, E. and STENT, G. S. Three star mutants of coliphage T2. J. Gen. Microbiol. **18**, 346–363 (1958).

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NOTE ON EFFECTS OF FEEDING GRASSHOPPERS TWO PATHOGENIC SPECIES OF BACTERIA SIMULTANEOUSLY[1]

JUNE M. STEPHENS[2]

Steinhaus (3) suggested that, among other factors, the combination of several pathogens might increase the effectiveness of microorganisms as disease agents of insects. This suggested that two bacterial species in combination might complement each other when fed to insects and a lowered LD_{50} would result. On the other hand, one organism might suppress the other and take precedence over it.

In conjunction with experiments on blood infection and determinations of LD_{50}'s, preliminary observations were made on the effects of feeding grasshoppers with a combination of two bacterial species simultaneously, in equal and varying proportions and under isolated and crowded conditions. The results of feeding grasshoppers with three combinations of two pathogenic species and some observations on blood infection are reported herein.

The following combinations of bacterial species orally pathogenic to grasshoppers were tested: (*a*) *Pseudomonas aeruginosa* (Schroeter) Migula and *Serratia marcescens* Bizio; (*b*) *P. aeruginosa* and a coliform-type bacterium; and (*c*) *S. marcescens* and the coliform-type bacterium.

For *P. aeruginosa*, strain 284-1A (1) was used. When inoculated into grasshoppers, this strain had an LD_{50} of about 20 bacteria; when fed, 28,000 to 60,000.

In feeding experiments testing the pathogenicity of *Bacillus cereus* Frankland and Frankland on the codling moth (5) many of the experimental insects became heavily infected with a red pigment-producing bacterium identified as *S. marcescens*. This organism was isolated from the apples on which the insects were fed. *S. marcescens* has been found in association with laboratory infections of a number of insect species (2). The LD_{50} of *S. marcescens* by injection into grasshoppers was about 14 bacteria; by feeding, about 28,000 bacteria. Hence *S. marcescens* was of the same relative order of pathogenicity for grasshoppers as *P. aeruginosa*. Blood samples to determine the degree of blood infection were taken from grasshoppers during the preliminary determination of the LD_{50}. About 67% of the grasshoppers that died from *S. marcescens* showed that the organisms had invaded the blood 1 to 3 days before death and these bacteria were present in the blood in approximately the same concentration reported for *P. aeruginosa* (6).

The coliform-type bacterium, strain 284-5A, was isolated from dead grasshoppers. The LD_{50} of this strain by inoculation into grasshoppers was about 50 organisms; by feeding, 600,000 organisms. Though feeding the organisms in 1% granular mucin (Wilson Laboratories, Chicago, Illinois) lowered the LD_{50} to about 84,000, this species is less virulent for grasshoppers than is *P. aeruginosa* or *S. marcescens*.

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The strains were combined by mixing equal volumes of cultures grown individually in Bacto nutrient broth (Difco Laboratories, Incorporated, Detroit, Michigan). Though the three strains did not vary greatly in the number of organisms per ml of culture, it was never possible to predict exactly the number of organisms in a given culture until actual counts were made. When the two cultures were combined and fed to grasshoppers, a range in proportion generally occurred.

Experimental grasshoppers, *Melanoplus bivittatus* (Say), from the same laboratory population were starved for one day, placed in half-pint cardboard containers, and then fed lettuce contaminated with the mixtures of organisms in the required dilutions to determine the LD_{50}. Experiments were carried on for 30 days. The LD_{50} was calculated by Probit analysis. At death grasshoppers were triturated and cultured; the species of organism occurring in pure culture or in decidedly greater number was considered to be the cause of death.

The results of feeding *P. aeruginosa* and S. *marcescens* to three groups of 50 grasshoppers each are as follows: In one group of grasshoppers confined individually and fed *P. aeruginosa* and *S. marcescens* in a ratio of 1:1, the deaths were all due to *S. marcescens* and the LD_{50} was 96,000 organisms. Blood samples taken from grasshoppers chosen at random and at random intervals showed that 7 out of 13 grasshoppers that later died from *S. marcescens* carried the bacteria in their blood. In the second group of grasshoppers confined individually and fed *P. aeruginosa* and *S. marcescens* in the ratio of 2:1, the LD_{50} was 66,000 total organisms; 83% of the total mortality was due to *P. aeruginosa*, the remainder to *S. marcescens*. In the 50 grasshoppers maintained in groups of 10, and fed *P. aeruginosa* and *S. marcescens* in the ratio of 3:1, 100% of the mortality was due to *P. aeruginosa*.

The LD_{50} was 69,000 total organisms when 50 grasshoppers maintained individually were fed *P. aeruginosa* and the coliform-type bacterium in a ratio of 3:1. This LD_{50} was higher than some LD_{50}'s obtained in feeding experiments with *P. aeruginosa* alone. Of 19 deaths, six were caused by *P. aeruginosa*, the others by the coliform-type bacterium.

Simultaneously, *P. aeruginosa* and the coliform-type bacterium were fed in the above ratio to 100 grasshoppers maintained in groups of 10 per container; the LD_{50} was 38,000 total organisms. Thirty-six deaths were caused by *P. aeruginosa* and seven by the coliform-type. This is the reverse of what happened in the preceding experiment and the LD_{50} was about one-half of that obtained when grasshoppers were maintained individually.

The LD_{50} was 63,000 total organisms when 50 grasshoppers were maintained individually and fed *S. marcescens* and the coliform-type bacterium in a ratio of 1:1. Eight of the deaths were caused by *S. marcescens* and six by the coliform-type bacterium. *S. marcescens* and the coliform-type bacterium similarly were not fed to grasshoppers kept in groups of 10.

Grasshoppers fed a combination of two pathogens generally die from an infection produced by one organism only and, as in grasshoppers fed one

pathogen (6), the blood was infected by the organism causing death, the other never being detected in the blood later than the first day after feeding. The organism taking precedence was generally that fed in greater numbers; when *P. aeruginosa* and *S. marcescens* were fed in equal numbers all the deaths were caused by *S. marcescens* but all deaths were caused by *P. aeruginosa* when it was fed in the greater proportion. The action by which one organism takes precedence and suppresses the other is as yet undetermined. However, in practically every case, grasshoppers died showing a pure culture of one organism only.

Crowding of grasshoppers and its attendant possibilities of cross-contamination and cannibalism apparently has more influence on lowering the LD_{50} than does the combination of pathogens or the ratio in which they are fed. This observation is in agreement with that of Steinhaus (4), who stated that crowding increased the incidence of disease among certain insects. Further experiments would be necessary to gain statistical evidence on the ability of these factors to lower the LD_{50}.

The results of combined feeding to grasshoppers of two pathogenic bacterial species indicate that the LD_{50} obtained is no lower than the LD_{50} of either alone. It may be concluded that, under the conditions of testing and in the combinations tested, no great advantage is obtained by feeding grasshoppers a combination of two pathogens.

1. Bucher, G. E. and Stephens, J. M. A disease of grasshoppers caused by the bacterium *Pseudomonas aeruginosa* (Schroeter) Migula. Can. J. Microbiol. **3**, 611–625 (1957).
2. Steinhaus, E. A. Insect microbiology. Comstock Publishing Co., Inc., Ithaca, N.Y. 1946.
3. Steinhaus, E. A. Potentialities for microbial control of insects. Agr. Food Chem. **4**, 676–680 (1956).
4. Steinhaus, E. A. Crowding as a possible stress factor in insect disease. Ecology, **39**, 503–514 (1958).
5. Stephens, J. M. Disease in codling moth larvae produced by several strains of *Bacillus cereus*. Can. J. Zool. **30**, 30–40 (1952).
6. Stephens, J. M. Occurrence of *Pseudomonas aeruginosa* (Schroeter) Migula in the haemolymph of grasshoppers after infection by feeding. Can. J. Microbiol. **4**, 191–193 (1958).

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CORRECTIONS

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Page 612. In Solution B, the quantity of $CuCl_2.2H_2O$ should read 0.269 mg.

NOTES TO CONTRIBUTORS

Canadian Journal of Microbiology

MANUSCRIPTS

General.—Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½×11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered. Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

Abstract.—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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